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(57) Abstract

The invention is directed to therapeutic use of fibronectin antagonists to inhibit microbial intracellular invasion of or adherence to host mammalian cells. Co–administration of the inhibitory compound with an antibiotic, such as penicillin, that inefficiently permeates mammalian cell membranes increases the efficacy of the antibiotic therapy.

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FIBRONECTIN ANTAGONISTS AS THERAPEUTIC AGENTS AND BROAD-SPECTRUM ENHANCERS OF ANTIBIOTIC THERAPY

This application claims the benefit of United States Provisional Patent Application, S/N 60/049124, filed June 10, 1997.

Background of the Invention

Streptococcus pyogenes, also known as group A streptococcus, is an important human pathogen capable of causing a wide variety of diseases. Before the turn of the century, this organism was frequently associated with systemic infections and puerperal sepsis (R. Atwater, *Am J. Hyg.*, 7, 343-369 (1927)). During the period from the early 1900s until the late 1980s, *S. pyogenes* was primarily associated with mild, self-limiting infections such as pharyngitis and impetigo and with the nonsuppurative sequelae, acute rheumatic fever and glomulonephritis.

Since 1987, however, there has been a dramatic, worldwide resurgence in the incidence of severe *S. pyogenes* infections including bacteremia, streptococcal toxic shock syndrome (STSS) and necrotizing fasciitis. The emergence of a particularly virulent clonal variant of serotype M1 streptococcus, termed M1inv+ for M type 1 invasive phenotype positive, was recently reported (D. LaPenta et al., *Proc. Nat'l Acad. Sci., U.S.A.*, **91**, 12115-12119 (1994); see also P. Schlievert et al., *J. Lab. Clin. Med.*, **127**, 13-22 (1996)). This variant supplanted earlier M1inv- strains sometime after 1988 as the major cause of uncomplicated disease, and widespread dissemination of this highly virulent clone seeded human populations with an organism possessing high potential to cause invasive disease (P. Cleary et al., *Lancet*, **321**, 518-521 (1992)). Other strains of *S. pyogenes*, such serotype M3 strains, have also been associated with contemporary serious *S. pyogenes* infection, including streptococcal toxic shock syndrome.

Despite the historical association of *S. pyogenes* with dangerous systemic infections, the prevailing epidemiology of *S. pyogenes* during the greater part of

the twentieth century has led researchers to view the organism as a model extracellular pathogen. Direct introduction of *S. pyogenes* into a wound or deeper tissue has been known to result in rapid disseminated disease (D. Stevens et al., *N. Eng. J. Med.*, **321**, 1-7 (1989)), but until very recently it was not known whether *S. pyogenes* has the inherent capacity to invade submucosal tissue in the absence of a mechanical breach of the epithelial barrier. In 1994, it was discovered that the bacterium can be internalized and survive within human epithelial cells (D. LaPenta et al., *Proc. Nat'l Acad. Sci., U.S.A.*, **91**, 12115-12119 (1994)). R. Greco et al. (*Res. Microbiol.*, **146**, 551-560 (1995)) also reported invasion of cultured epithelial and endothelial cells by *S. pyogenes* type M5 and M12. Proteins M6 and F1 were reported to be required for efficient invasion of *S. pyogenes* into cultured epithelial cells (J. Jadoun et al., pp. 511-515 *in* T. Horaud et al., (Ed.) Streptococcus and the Host, Plenum Press, New York (1997)); and the fibronectin-binding protein of *Streptococcus pyogenes*, SfbI, was shown to be involved in the internalization of *S. pyogenes* by epithelial cells. G. Molinari et al., *Infect. Immun.*, **65**, 1357-1363 (1997)). Intracellular streptococci have been observed in tonsils removed from children with a history of recurrent pharyngitis (A. Osterlund et al., *Laryngoscope*, **107**, 640-646 (1997)).

Internalization into host cells allows the organism to evade the immunological defenses of the host. Significantly, the recently discovered M1inv+ clonal variant studied by LaPenta et al. is measurably more invasive than either the M1inv- or M12 strains, with internalization into epithelial cells as efficient as that observed previously for *Salmonella typhimurium* (J. Kusters et al., *Infect. Immun.*, **61**, 5013-5020 (1993)) and *Listeria monocytogenes* (J. Gaillard et al., *Cell*, **65**, 1127-1141 (1991)). *Streptococcus agalactiae* (also known as group B streptococcus) (C. Rubens et al., *Infect. Immun.*, **60**,5157-5163 (1992)) and *Streptococcus pnueumoniae* (U. Talbot et al., *Infect. Immun.*, **64**, 3772-3777 (1996)) have also been found to invade human respiratory epithelial cells. All three streptococcal species are suspected of utilizing invasion of epithelial cells in order to gain access to deeper tissues and to the bloodstream. The biomolecular basis for bacterial internalization, however, has yet to be fully elucidated.

Adding to the gravity of this epidemiological shift toward *S. pyogenes* infections of greater frequency and severity are recent reports indicating that antibiotic therapies used for the treatment of streptococcal infections are often inadequate. Penicillin has historically been the most frequently prescribed drug for streptococcal infections. It is still considered the most effective antibiotic, even though continued carriage of the organism despite penicillin treatment is now well-documented. Once inside a mammalian cell, the streptococci are able to avoid exogenously administered penicillin (D. LaPenta et al., *Proc. Nat'l. Acad. Sci., U.S.A.*, **91**, 12115-12119 (1994)). Even in the context of mild pharyngitic infections, penicillin treatment does not successfully remove the pathogen. For example, viable streptococci can be cultured from the throats of up to 50% of patients who have been treated with penicillin for 10 days, although most treated patients exhibit no overt symptoms of infection. Recurrent infections are common.

A strain such as M1inv+ that efficiently invades mammalian cells is more likely than others to be both carried and shed after penicillin therapy. Indeed, asymptomatic human infections appear to be the reservoir for more severe *S. pyogenes* infections that occur, through contamination of a wound or even minor breaks in the integument, as a result of self-inoculation or direct contact with others who shed the organism. Clearly, as the fraction of individuals who shed M1inv+ streptococci increases in a population, the probability of wound contamination increases. Although penicillin therapy cannot reach internalized streptococci, the recommended antibiotic treatment for serious infections remains a combination of a β -lactam (penicillin or ceftriaxone) and a protein synthesis inhibitor (clindamycin or erythromycin); a more effective antibiotic regimen is not currently available. For treatment of carriage, a therapeutic regimen comprising a short course of rifampin in conjunction with penicillin has been prescribed; Oral clindamycin has also been shown to have some beneficial effect (see M. Gerber et al., *Pediatr. Inf. Dis. J.*, **13**, 576-579 (1994), for details regarding dosages).

The observed persistence of *S. pyogenes* after penicillin therapy is thus likely due, at least in part, to their recently discovered ability to be internalized and persist in epithelial cells. These bacteria have remained, genetically,

exquisitely sensitive to penicillin. However, penicillin penetrates mammalian cell membranes poorly. What is needed, therefore, is a therapy that improves the efficacy of penicillin treatment by interfering with the microbe's attempts to isolate itself from interaction with the antibiotic.

Summary of the Invention

The present invention provides a therapeutic composition useful for treating a mammal, preferably a human, infected by a pathogenic microbe. The composition of the invention contains an inhibitory compound in an amount effective to inhibit adherence to or invasion of a mammalian cell by the pathogenic microbe, at least one antibiotic effective to treat the pathogenic microbe, and a pharmaceutically acceptable carrier. In a preferred embodiment, the inhibitory compound is an host cell integrin antagonist or a microbial invasin antagonist. In a particularly preferred embodiment, the composition includes a fibronectin antagonist that binds to an $\alpha 5\beta 1$ integrin.

The invention further provides a method for treating a mammal infected with a microbial pathogen that includes administering to the mammal the inhibitory compound of the invention. Optionally, the method of treatment further includes administering an effective amount of at least one antibiotic effective to treat the pathogenic microbe. For example, to treat infection by the pathogenic microbe *S. pyogenes*, the inhibitory compound is advantageously administered in conjunction with penicillin or erythromycin.

The invention also provides a method for identifying an inhibitory compound useful in the compositions and treatment methods of the invention. In one embodiment, the method for identifying inhibitory compounds effective to inhibit adherence to or invasion of a mammalian cell by a pathogenic microbe includes (a) exposing a mammalian cell culture to a pathogenic microbe in the presence of a candidate inhibitory compound and at least one stimulatory factor that stimulates adherence to or invasion of the mammalian cell by the pathogenic microbe, for a time effective to allow adherence to or invasion of the mammalian cell by the pathogenic microbe; and (b) assaying the cell culture to determine the presence of internalized or adhered pathogenic microbe.

In an alternative embodiment of the method, the steps for identifying inhibitory compounds effective to inhibit adherence to or invasion of a mammalian cell by a pathogenic microbe include (a) exposing a mammalian cell culture to a detectably labeled pathogenic microbe in the presence of a candidate inhibitory compound and at least one stimulatory factor that stimulates adherence to or invasion of the mammalian cell by the pathogenic microbe, for a time effective to allow adherence to or invasion of the mammalian cell by the pathogenic microbe; (b) removing unbound and uninternalized pathogenic microbe from the mammalian cell culture; and (c) detecting the presence or absence of a detectable label in the mammalian cell culture. The absence of a detectable label is indicative of an inhibitory compound that is effective to inhibit adherence to or invasion of the mammalian cell by the pathogenic microbe. Optionally, the method can further include (d) contacting the mammalian cell culture with a fluorescence quenching agent; and (e) detecting the presence or absence of fluorescence in the mammalian cell culture. The absence of fluorescence in step (e) is indicative of an inhibitory compound that is effective to inhibit invasion of the mammalian cell by the pathogenic microbe.

In a preferred embodiment of the method for identifying inhibitory compounds, the candidate inhibitory compound is a fibronectin antagonist that binds to a fibronectin receptor, such as an $\alpha 5\beta 1$ integrin.

Brief Description of the Drawings

Figure 1 shows ECM protein-mediated intermolecular interactions between host cell integrins and microbial invasions that may lead to internalization of pathogenic microbes by host cells (upper pathway); these interactions can be disrupted or blocked by various inhibitory compounds according to the present invention (lower pathway), such as ECM mimics (1) and anti-integrin antibodies (2) that bind to host cell integrins; integrin mimics (3), invasin mimics (4) and anti-ECM protein antibodies (5) that bind to ECM proteins; and ECM mimics (6) and anti-invasin antibodies (7) that bind to microbial invasions.

Figure 2 shows the effects of selected agonists on (a) internalization and (b) adherence of $M1^+$ and $M1^-$ streptococci using cultured epithelial cells.

Figure 3 shows invasion inhibition of cultured epitheilal cells by anti-fibronectin antibody in the presence of selected agonists.

Figure 4 shows binding of ^{125}I-labeled fibronectin to wild-type (•) and $M1^-$ streptococci (♦).

Figure 5 shows the effects of anti-integrin monoclonal antibodies on bacterial invasion of cultured epithelial cells.

Figure 6 shows invasion inhibition of cultured epithelial cells by (a) anti-integrin β1 monoclonal antibodies and (b) anti-integrin α5β1 monoclonal antibodies.

Figure 7 shows the effects of selected peptides on internalization of $M1^-$ streptococci by cultured epithelial cells.

Figure 8 shows the effects of integrin antagonists on intracellular invasion by *S. pyogenes*; shaded bar: DMP757 (an αIIbβ3 antagonist); open bar: XT199-4 (an αvβ3 antagonist); hatched bar: no drug (control).

Figure 9 shows the effect of (S)-2-[(2,4,6-trimethylphenyl]amino-3-[[7-benzyloxy carbonyl-8-(2-pyridinylaminomethyl)-1-oxa-2,7-diazaspiro-[4,4]-non-2-en-3-yl] carbonylamino] propionic acid, referred to herein as "SJ749-1" (unbroken line) on intracellular invasion by *S. pyogenes*; the broken line represents a control experiment.

Figure 10 shows the effects of SJ749-1 (an α5β1 antagonist) on growth of *S. pyogenes* (solid line) and adherence of A549 cells to tissue culture wells (broken line).

Figure 11 shows the effect of the aniplatelet compound ticlopidine on intracellular invasion by *S. pyogenes*.

Figure 12 shows bacterial adherence to and entry into primary cultures of tonsillar epithelial cells, for both $M1^+$ and $M1^-$ streptococci.

Figure 13 shows agonist-dependent invasion of PTE cells by $M1^+$ streptococci.

Figure 14 shows inhibition of streptococcal invasion of PTE cells by integrin antagonists.

Figure 15 shows inhibition of streptococcal invasion of cultured epithelial cells by kinase inhibitors.

Figure 16 shows that anti-α5β1 integrin SJ749-1 can increase the sensitivity of *S. pyogenes* to penicillin in epithelial cell culture.

Detailed Description of the Invention

Interactions between eukaryotic cells and components in the extracellular matrix are mediated by specific cellular receptors, of which integrins are the best characterized. Integrins are cell surface receptors that bind to extracellular matrix ligands or other cell adhesion ligands and thereby mediate cell-cell and cell-matrix adhesion processes. These receptors are typically composed of heterodimeric transmembrane glycoproteins containing α- and β-subunits. Integrin subfamilies contain a common β-subunit combined with different α-subunits to form adhesion receptors with unique specificity.

Extracellular matrix (ECM) proteins are known to bind integrins and thereby facilitate cellular adhesion processes. ECM proteins include, among others, fibrinogen, fibronectin, laminin, vitronectin, collagen, elastin, chondronectin, entactin/nidogen and tenascin. Fibronectin and laminin are high-molecular weight, extracellular glycoproteins present in blood and the extracellular matrix of numerous tissues.

Cell-matrix and cell-cell adhesion processes in eukaryotes can be altered by various protein, peptide, monoclonal antibodies, and peptidomimetic compounds (e.g., WO 96/38426; EP 478363; EP 478328; EP 525629; WO 95/14683; EP 4512831; U.S. Patent No. 5,635,477 (June 3, 1997); and S. Mousa et al., *Circulation*, 93, (1996), all of which are incorporated herein in their entireties; and references cited therein). Some of these compounds, known as integrin antagonists, bind to an integrin receptor. For example, the peptidomimetic compounds reported in WO 96/38426 compete with fibronectin for binding to integrin receptors and thereby inhibit platelet aggregation. These integrin antagonists have reported utility for the treatment and prevention of thromboembolic disorders and autoimmune diseases which involve cell adhesion processes such as inflammation, bone degradation, and rheumatoid arthritis.

Several integrin antagonist drugs are currently under development for the treatment of inflammation, thromboembolic disorders and for increasing the biocompatibility of implantable prosthetic devices. For example, Mousa et al. (*Circulation*, **93**, 537-543 (1996)) describe DMP 728, a low molecular weight GPIIb/IIIa receptor antagonist that may have therapeutic potential as an intravenous or oral antithrombotic agent in coronary and carotid artery thromboembolic disorders. A number of these drugs (e.g., REOPRO, TIROFIBAN, and INTEGRILIN are already approved for therapeutic use, and several others are in clinical trials (S. Mouse et al., *Drugs of the Future*, **21** (11): 1141-1150 (1996)).

A number of bacteria capable of causing invasive diseases are known to be proficient at invading cultured eukaryotic cells (e.g., M. Maurin et al., *Drugs*, **52**, 45-59 (1996)). For example, several bacterial pathogens, including *Streptococcus pyogenes, Streptococcus pneumoniae, Streptococcus agalactiae, Staphylococcus aureus, Chlamydia trachomatis, Neiserria gonorrhoeae, Salmonella typhii,* and *Enterococcus faecalis,* are known to cause recurrent infections, to produce carrier states, or to persist following treatment of the initial infection, even though they still exhibit sensitivity to the antibiotic in question *in vitro*. All of these species can be internalized and survive inside epithelial cells and/or professional phagocytes. For some, the intracellular state is an obligatory stage in their life cycle. These intracellular pathogens colonize mucosal tissue either by adherence to the extracellular matrix (ECM) or direct interaction with host cell receptors.

Adherence of bacteria to nonphagocytic eukaryotic cells is a multifactorial process that can involve interactions between and among eukaryotic and bacterial receptors, extracellular factors and intracellular signaling pathways of both pathogen and host. Often it occurs in successive stages which can induce physiological changes in the microbe or the infected cell, and in some cases triggers internalization of the microbe. Intracellular infection is a dynamic process whereby bacteria cycle between intracellular and extracellular states. Interruption of this cycle would prevent internalization of infectious agents that depend on interactions with integrin type receptors.

Some microorganisms adhere to host cells by exploiting components of the host cells' adhesion system. These microorganisms express cell surface adhesins that mediate microbial adhesion to the extracellular matrix of host tissues. Extracellular matrix proteins (ECM proteins) that are recognized by various microbial cell surface receptors include fibronectin, fibrinogen and vitronectin, as well as collagen and laminin. These ECM proteins may serve as bridges to tissue receptors or activate adhesins on the microbe surface that can then bind to such receptors.

For example, *S. aureas*, *S. pneumoniae*, *C. albicans*, and *S. pyogenes* have integrin receptors on their surface which can bind to human adhesion and ECM proteins. Vitronectin and fibronectin can facilitate intracellular invasion by *N. gonorrhoeae* and *M. bovis*, respectively, and invasion by *M. leprae* can be stimulated by microbial binding of fibronectin or, as suggested by a recent report, laminin (A. Rambukkana et al., *Cell*, **88**, 803-831 (1997)). Fibronectin-binding adhesins are present on the cell surfaces of other pathogenic bacteria as well (e.g., *S. aureus*, group A, C and D streptococci, *Prevotella (Bacteriodes) intermedia*, and *Porphyromonas gingivalis*) and fungi (e.g., *C. albicans* and *A. fumigatus* (J. Patti et al., *Ann. Rev. Microbiol.*, **48**, 585-617 (1994)).

In other cases, bacterial adhesion to and/or invasion of eukaryotic host cells occurs via direct contact between cell surface proteins (i.e., is not mediated by ECM proteins). For example, the abilities of *Listeria* and *Yersinia* to invade cultured cells have been shown to be dependent upon the expression of specific, bacterial cell-surface proteins. Host cell integrins are utilized by enteropathogenic *Yersinia* for entry into mammalian cells. *Yersinia* internalization is mediated by an invasin, a bacterial cell surface protein with a high affinity to β1 integrins. Invasin binding to integrins results in activation of host cell signal transduction pathways which leads to actin-mediated "zipper phagocytosis" of adherent bacteria (see, e.g., B. Finlay et al., *Microbiol. Molec. Biol. Rev.*, **61**, 136-169 (1997)); R. Isberg et al., *Cell*, **60**, 861-871 (1990); and G.T. Van Nhieu et al., *J. Biol. Chem.*, **266**, 24367-24375 (1991)). At least one other bacterial pathogen, *Mycobacterium avium-Mycobacterium intracellulare*, and one fungal pathogen, *C. albicans*, have been shown to express integrin receptors on their cell surfaces.

It is an object of the present invention to block intracellular invasion of host mammalian cells, preferably mammalian nonphagocytic cells, by microbial pathogens. Typically, internalization is initiated by adherence of an invasive microbe to the mammalian host cell that is mediated by a host cell integrin. Binding to the integrin generates signals that lead to internalization of the microbial pathogen into an antibiotic impermeable vacuole or compartment in the host cell.

A compound blocks or inhibits microbial invasion of a host mammalian cell if the host mammalian cells exhibit reduced levels of internalization of the microbe in the presence of the compound. By preventing intracellular invasion by microbial pathogens, an inhibitory compound useful in the present invention disrupts the intracellular/extracellular cycling of the invasive microbial pathogen. Additionally, administration to a mammal of an inhibitory compound described herein enhances the efficacy of antibiotics that poorly penetrate the host cell membrane (e.g., penicillins, cyclosporins, gentamicin, and isoniazid, R. Prokesch et al., *Antimicrobial Agents and Chemotherapy*, **21**, 373-380 (1982)), since more microbial pathogens remain outside the host mammalian cell. Detailed evaluations of antibiotic (penicillin and others) penetration of mammalian cells are described by M. Maurin et al., in "Antimicrobial Agents and Intracellular Pathogens," CRC Press, Inc. N4, 23-37 (1993), incorporated herein by reference.

It is a further object of the invention to inhibit or disrupt adherence of microbial pathogens to a host cell membrane. In addition to preempting subsequent intracellular invasion, inhibition of adherence prevents aggregation and clustering of bacteria on the host cell surface, making it more likely that the bacterial cell will come into to contact with an extracellular antibiotic. Moreover, inhibitory compounds that block adherence of the bacterial cell to epithelial tissues could prevent persistent colonization of host tissue even without concurrent administration of antibiotics. Unable to adhere, bacteria would be removed from host tissue by the cleaning action of beating cilia, movement of fluids across mucosal surfaces, and innate phagocytic defenses. A compound inhibits adhesion of a pathogenic microbe to a host mammalian cell

when the microbe exhibits reduced levels of binding to the surface of the host cell in the presence of the compound.

Any compound that interferes with the adherence to or invasion of the mammalian cell by the pathogenic microbe, thereby preventing or reducing internalization of the pathogenic microbe by the host cell, constitutes an inhibitory compound that can be used in the therapeutic treatment methods and compositions of the invention. Preferably, an inhibitory compound useful in the treatment methods and therapeutic compositions of the invention inhibits invasion of the host cell by the pathogenic microbe. More preferably, the inhibitory compound can, but need not, additionally inhibit adherence of the pathogenic microbe to the host mammalian cell. It is to be understood that a pathogenic microbe may still adhere to the surface of a mammalian cell in the presence of an inhibitory compound useful in the present invention, provided the ability of the microbe to invade the host cell is nonetheless reduced or eliminated by the inhibitory compound. An important feature of the inhibitory compound as used in the present invention is that fewer pathogenic microbes are internalized by a mammalian host cell in its presence compared to the number of pathogenic microbes internalized in its absence.

The inhibitory compound can be a naturally occurring compound or a synthetic molecule. In a preferred embodiment, the inhibitory compound is selected from the group consisting of a cyclic peptide, preferably a cyclic RGD peptide, a non-peptide including a peptidomimetic non-peptide, and an antibody. Preferably, the inhibitory compound is a synthetic non-peptide. More preferably, the inhibitory compound is synthetic peptidomimetic that functions as an integrin antagonist, preferably a $\alpha 5\beta 1$ – specific integrin antagonist. Inhibitory compounds that are useful in the compositions and treatment methods of the invention are readily identifiable by one of skill in the art by employing the screening and assay methods as set forth herein, including those disclosed in the following examples.

It should be understood that inhibition of adherence to or invasion of the mammalian cell by the pathogenic microbe, as effected by an inhibitory compound of the invention, is not limited to any particular cellular or biochemical mechanism or mode of operation. Without limiting the scope of the

invention, the inhibitory compounds can therefore be exemplified with reference to their functional characteristics and their known or predicted mechanism of operation. For example, where interactions between bacteria and mammalian host cells are mediated by ECM proteins, inhibitory compounds useful in the present invention include those that prevent internalization of bacteria by the mammalian host cells by successfully competing with ECM proteins for binding to either host cell integrins or receptors present on the surface of bacteria, referred to herein as "invasins." As used herein, the term "invasin" is to be broadly understood as including any protein present on a microbial cell surface that mediates internalization of the microbe by the host mammalian cell, and includes, but it not limited to, microbial ECM protein receptors, microbial integrin-like receptors and mimics, microbial adhesins, and the like. Inhibitory compounds can include compounds that bind to host cell integrins, that bind to one or more ECM proteins, or, additionally or alternatively, that bind to a microbial invasin that interacts with an ECM protein. These compounds can, for example, be monoclonal or polyclonal antibodies, linear or cyclic peptides, preferably cyclic peptides containing the tripeptide arginine-glycine-aspartate (RGD), or non-peptides, including peptidomimetic compounds. See, e.g., compounds disclosed in WO97/33887 (Jadhav et al., published 18 September 1997). More preferred inhibitory compounds include analogs or mimics of fibronectin or laminin. Particularly preferred are those inhibitory compounds that compete with the binding of fibronectin to host cell integrins. In certain preferred embodiments of the composition and methods of the invention, the inhibitory compound is a fibronectin antagonist, i.e., a compound that competes with the binding of fibronectin to a fibronectin receptor, such as an $\alpha 5\beta 1$ integrin. For example, representative non-peptide fibronectin antagonists that interfere with fibronectin binding to $\alpha 5\beta 1$ integrin include (S)-2-[(2,4,6-trimethylphenyl)sulfonyl]amino-3-[[7-benzyloxy carbonyl-8-(2-pyridinylaminomethyl)-1-oxa-2,7-diazaspiro-[4,4]-non-2-en-3-yl] carbonylamino] propionic acid, referred to herein as "SJ749-1," and its analogs as shown in Table 3, below. Anti-integrin antibodies are also preferred inhibitory compounds, as are anti-ECM antibodies, such as anti-fibronectin

antibodies and anti-laminin antibodies, where such antibodies interfere with the binding of an ECM protein to a host cell integrin or a microbial invasion.

Representative compounds that inhibit microbial adherence to or invasion of a mammalian cell by interfering with the interactions between a microbial invasin and an ECM protein can include, in addition to those that bind sites on the ECM protein, compounds that bind to microbial invasins. Examples of microbial invasins include, but are not limited to, bacterial M protein, protein F, SFbI, SFbII, Fbp54 and other cell wall associated fibronectin-binding proteins. For example, antibodies to M protein that inhibit streptococcal adherence to or invasion of host epithelial or endothelial cells are inhibitory compounds of this type.

Inhibitory compounds useful for the treatment methods and compositions of the invention can include the tripeptide sequence arginine-glycine-aspartic acid (RGD), provided they interfere with microbial adherence to or invasion of a host mammalian cell. The RGD sequence is present in numerous adhesive proteins, including fibronectin, and is an essential determinant in the binding of adhesive proteins by the integrin class of cellular receptors. Some RGD-containing peptides, however, stimulate rather than prevent host cell internalization of pathogenic microbes; these stimulatory compounds are not considered inhibitory compounds according to the invention.

Alternatively or additionally, where microbial pathogenesis results from direct contact between the microbial cell and the mammalian cell (i.e., that is not mediated by an ECM factor of the mammalian host), inhibitory compounds useful in the present invention include those that interfere with interactions between the cells so as to prevent adherence to or invasion of the mammalian cell by the microbial pathogen. For example, where bacterial adhesion to and/or invasion of host cells occurs via direct contact between cell surface proteins, an inhibitory compound useful in the invention can interfere with this contact by binding to the host and/or the microbial cell surface protein involved in the intercellular interaction.

Moreover, it seems likely that ECM protein-mediated intracellular invasion by pathogens occurs via activation of host cell signal transduction pathways in a manner similar to that caused by *Listeria* and *Yersinia*, which

enter host cells via direct contact between bacterial and host cell surface proteins. Integrin-mediated uptake of microorganisms can involve, in addition to microbial engagement of receptors, host cell signal transduction via integrin clustering or conformational changes and phosphorylation of intracellular macromolecules and reorganization of the host cell cytoskeleton. Thus, compounds that prevent the activation of this signal transduction pathway or interfere with reorganization of the host cell cytoskeleton can also inhibit adherence to or invasion of the mammalian cell by the microbial pathogen. Inhibitory compounds useful in the methods and compositions of the present invention therefore include compounds that interfere with transduction of a signal through the membrane of the host cell that is otherwise initiated by interactions with a microbial pathogen, whether or not mediated by an ECM protein, such as kinase inhibitors, and compounds that block the cytoskeletal and other physiological changes required to initiate endocytosis such as those, for example, that impede actin polymerization, e.g., cytochalasin D (Schwartz et al., *Ann. Rev. Cell. Dev. Biol.*, **11**, 549-599 (1995). Examples of kinase inhibitors that constitute inhibitory compounds of the invention include staurosporine (I. Rosenshine et al., *Methods Enzymol.*, **236**, 467-476 (1994)) genistein (I. Rosenshine et al., *Methods Enzymol.*, **236**, 467-476 (1994)) and wortmannin (I. Nakamura et al., *FEBS Lett.* **361**, 79-84 (1995).

The present invention thus provides a method for treating microbial infection or disease in a mammal, preferably a human, comprising administration of a therapeutic composition containing compound that inhibits adherence to or invasion of host cells of the mammal by a microbial pathogen, and, optionally, a pharmaceutically acceptable carrier. The microbial pathogen can, for example, be a bacterium, fungus, a protozoan, or a virus. Preferably, the microbial pathogen is a bacterium. Any microbial pathogen whose infectious process involves adherence to or invasion of host mammalian cells, preferably nonphagocytic cells of the host, more preferably host epithelial or endothelial cells, is contemplated by the treatment method of the present invention. Preferably, the pathogenic microbe binds a host cell integrin or an extracellular matrix protein or both. The therapeutic composition can be administered to treat an existing microbial infection, or prophylactically in the case of potential or

suspected exposure to an infectious microbial pathogen. Preferably, the therapeutic composition is administered to treat an existing infection. The method is particularly well-suited for treatment of systemic infections, deep tissue infections, necrotizing fasciitis, endocarditis, lung infections, and persistent recurring microbial infections of any type.

In a preferred embodiment, the method is effective in treating infection by *Streptococcus*, *Enterococcus*, *Yersinia*, *Salmonella*, *Listeria*, *Shigella*, *Neisseria*, *Mycobacterium*, *Staphylococcus*, *Prevotella*, *Porphyromonas*, *Helicobacter*, *Legionella*, *Candida*, *Histoplasma* and *Aspergillus*. More preferably, the method is effective in treating infection by group A, B, C and D streptococci, *Streptococcus pnueumoniae*, *Enterococcus*, *Yersinia*, *Salmonella typhimurium*, *Listeria monocytogenes*, *Shigella*, *Neisseria gonorrhoeae*, *Mycobacterium leprae*, *Staphylococcus aureus*, *Prevotella (Bacteriodes) intermedia*, *Porphyromonas gingivalis*, *Helicobacter*, *Legionella* or the fungi *Candida albicans*, *Histoplasma*, or *Aspergillus fumigatus*; most preferably, the method is effective for treating infection by *S. pyogenes*.

The mammalian host cells to which the microbial pathogens adhere, or which they invade, are preferably, but need not be, nonphagocytic cells; more preferably, the host mammalian cells are epithelial or endothelial cells.

The inhibitory compound used in the treatment method and composition of the present invention inhibits adherence to or invasion of a cell of the mammal by the pathogenic microbe and includes, but is not limited to, a compound selected from the class of compounds comprising integrin antagonists, invasin antagonists, and compounds that inhibit integrin-initiated cellular signaling processes and cytoskeletal rearrangements associated with host cell internalization of pathogenic bacteria, Fig. 1 shows ECM-mediated internalization of a pathogenic microbe, together with integrin antagonists and invasin antagonists that inhibit or prevent internalization of the invasive pathogenic microbe according to the invention. Determination of whether a compound inhibits adherence to and/or invasion of host cells of the mammal by a microbial pathogen, and thus constitutes an "inhibitory compound" for use in the method and compositions of the invention, can, for example, be made by

carrying out the adherence and internalization assays described herein, including Examples I, III, IV and VII.

As used herein, the term "integrin antagonist" is to be broadly understood as including any compound that interferes with an interaction between a host cell integrin and, in the case of ECM protein-mediated interactions with pathogenic microbes, an ECM protein. In the case of intercellular interactions that are not mediated by an ECM protein, the term "integrin antagonist" includes any compound that interferes with a direct interaction between a host cell integrin and microbial invasin. Accordingly, inhibitory compounds useful in the present invention include integrin antagonists that inhibit adherence to or invasion of a cell of the mammal by the pathogenic microbe. The term "integrin antagonist" includes compounds that compete with a natural ligand of a mammalian host cell integrin for binding to that integrin. For example, the term includes structural or steric analogs of a natural integrin ligand or the ligand-binding site on an integrin; anti-integrin antibodies, including antibodies to particular integrin subunits such as $\beta 1$, $\beta 3$, or $\alpha 5$ antibodies or anti-ligand antibodies, that bind to a site on the integrin or the ligand, respectively, so as to interfere with binding of the ligand to the integrin, and any other molecule that competes with ligand binding to an integrin. The term integrin antagonist is to be broadly construed as including compounds commonly referred to as anti-integrins, integrin mimics, integrin analogs, and so on, including ECM antagonists such as fibronectin antagonists and laminin antagonists, and anti-integrin and anti-ECM monoclonal and polyclonal antibodies. Compounds known and used as platelet inhibitors, platelet antagonists, anti-platelet agents, anti-thrombotics, anti-neoplastics, anti-cancer agents, and antimetastasis agents and the like can constitute inhibitor compounds useful in the invention provided they inhibit adherence to or invasion of a cell of the mammal by the pathogenic microbe. A compound that chemically alters a host cell integrin or ECM protein so as to interfere with the interactions between the host cell integrin and the ECM protein is also considered an integrin antagonist for purposes of the present invention.

The term "invasin antagonist" is to be broadly understood as including any compound that interferes with an interaction between a microbial invasin and, in the case of ECM protein-mediated interactions with mammalian host

cells, an ECM protein. In the case of intercellular interactions that are not mediated by an ECM protein, the term "invasin antagonist" includes any compound that interferes with a direct interaction between a host cell integrin and a microbial invasin. Accordingly, inhibitory compounds useful in the present invention invasin antagonists that inhibit adherence to or invasion of a cell of the mammal by the pathogenic microbe. The term "invasin antagonist" includes compounds that compete with a natural ligand of a microbial invasin for binding to that ligand. For example, the term includes structural or steric analogs of a natural invasin ligand or the ligand-binding site on an invasin; anti-invasin antibodies or anti-ligand antibodies, that bind to a site on the invasin or the ligand, respectively, so as to interfere with binding of the ligand to the invasin, and any other molecule that competes with ligand binding to an invasin. The invasin antagonist includes a compound that chemically alters a microbial invasin or ECM protein so as to interfere with the interactions between the microbial invasin and the ECM protein.

Inhibitory compounds that inhibit adherence to or invasion of a cell of the mammal by the pathogenic microbe can include compounds that inhibit integrin-initiated cellular signaling processes include, for example, kinase inhibitors, such as staurosporine, genistein, and wortmannin. Also included are compounds that interfere with cytoskeletal rearrangements associated with host cell internalization of pathogenic bacteria and other endocytotic processes. For example, cytochalasin D, colchicine, and certain microbial toxins have been shown to impede actin polymerization.

The invention provides a method for treating a mammal infected by a pathogenic microbe comprising administering to the mammal a therapeutic composition comprising an inhibitory compound in an amount effective to inhibit adherence to or invasion of a cell of the mammal by the pathogenic microbe. Administration of the therapeutic composition can take any convenient form. The formulations include those suitable for oral, rectal, topical, nasal, ophthalmic, or parenteral (including subcutaneous, intramuscular and intravenous) administration, all of which may be used as routes of administration for practicing the present invention. Oral or intramuscular administration is

preferred. The therapeutic composition can also be administered topically, for example to treat a wound or skin condition, or for absorption through the skin.

The therapeutic composition can be formulated in any convenient manner as prescribed by the selected mode of administration, for example as a tablet, capsule, suppository, or injection. Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets, tablets or lozenges, each containing a predetermined amount of the potentitating agent as a powder or granule, as liposomes, or as a suspension in an aqueous liquor or nonaqueous liquid such as a syrup, an elixir, an emulsion or a draught. Particularly preferred are oral formulations that take the form of a mouthwash, gargle, throat lozenge, and the like. Formulations suitable for topical use include liquids, lotions, salves, gels, cremes, and the like. Methods for introducing the formulations *via* each of these routes are well-known in the art.

Dose of the inhibitory compound will vary depending on the particular route of administration used. In general, for systemic treatments, e.g., those administered intramuscularly or intravenously, an overall dose range of about 1-6 mg/kg body weight, preferably 2-5 mg/kg body weight is contemplated. The inhibitory compound can be administered via nebulizer, in which event the dosage would be expected to be similar to that used for prophylactic administration of pentamidine (for a 70 kg person, about 300 mg in 6 mL water, administered once every four weeks) (K. Goa et al., *Drugs*, 33, 242-0258 (1987); Physicians Desk Reference, 50th Ed., Medical Economics Co., Montvale, N.J., 1996). A similar concentration of the inhibitory compound (but a lower dosage) could be administered nasally or applied topically to the oral mucosa in the treatment of upper respiratory tract infections. For topical administration, for example, about a 0.1-1% suspension of the inhibitory compound is preferably contemplated. A typical dosage for ophthalmic administration would be about 0.5-2 mg/mL (e.g., D. Perrine et al., *Antimicrob. Chemother.*, 39, 339-342 (1995)).

One of skill in the art will appreciate that known dosages for various therapeutic integrin antagonists, anti-integrins, fibronectin antagonists, laminin antagonists, anti-neoplastics, anti-cancer agents and antimetastasis agents can be

used as a basis for determining an effective dose of the inhibitory compound of the present invention in accordance to routine pharmaceutical practice.

The formulations may be conveniently presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active compound into association with a carrier which constitutes one or more accessory ingredient. In general, the formulations are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into the desired formulations.

In addition to the aforementioned ingredients, the formulations of this invention may further include one or more accessory ingredient(s) selected from diluents, buffers, flavoring agents, binders, disintegrants, surface active reagents, thickeners, lubricants, preservatives, (including antioxidants) and the like.

A preferred embodiment of the invention provides a method for treating infection or disease in a mammal, preferably a human, comprising administration of a therapeutic composition containing an effective amount of a compound that inhibits adherence to or invasion of host cells of the mammal by a microbial pathogen, and administration of an effective amount of at least one antibiotic effective to treat the microbial pathogen. The inhibitory compound and the antibiotic can be administered together in single formulation or a plurality of individual formulations, each of which, optionally, includes a pharmaceutically acceptable carrier. If administered separately, they can be administered either contemporaneously or at different times during the treatment period.

Preferably, the antibiotic used in the present treatment method is effective to kill or disable the suspected infectious microbial pathogen. The antibiotic is administered to the mammal in an amount effective to cause such killing or disabling. One of the skill in the art will readily appreciate that the effective amount depends upon the method of administration. The antibiotic can be an antibacterial, antiviral, antifungal, or antiprotozal compound. Preferred antibiotics include β-lactams (e.g., penicillin), cephalosporins, macrolides (e.g., erythromycin and clindamycin), and aminoglycosides (e.g., gentamicin). Penicillin and erythromycin are particularly preferred. Dosages and courses of treatment for antibiotics are well known in the medicinal and pharmaceutical

arts. It is contemplated that an effective amount of an antibiotic used in the present compositions or in the present method is comparable to the dose administered in standard therapeutic applications for the particular antibiotic, when administered independently. One of skill in the art will appreciate that standard antibiotic dosages form a convenient basis from which to determine effective amounts for purposes of the invention in accordance with routine pharmaceutical development.

An alternative method provided by the invention includes treatment of human infection or disease comprising administration of a therapeutic composition containing an effective amount of a compound that inhibits adherence to or invasion of host cells of the mammal by a microbial pathogen; an effective amount of an antibiotic effective to treat the microbial pathogen; and an effective amount of a protein synthesis inhibitor, preferably clindamycin or erythromycin. Instead of or in addition to a protein synthesis inhibitor, a bacterial RNA synthesis inhibitor, such as rifampin, may be included in the therapeutic composition.

Another alternative method provided by the invention includes treatment of human infection or disease comprising administration of a therapeutic composition containing an effective amount of a compound that inhibits adherence to or invasion of host cells of the mammal by a microbial pathogen; an effective amount of an antibiotic effective to treat the microbial pathogen; and an effective amount of an inhibitor of a bacterial or host cell enzyme capable of degrading or otherwise inhibiting the activity of the antibiotic. For example, when the antibiotic is penicillin (a β-lactam), a penicillinase (β-lactamase) inhibitor, such as clavulanic acid, can be included in the therapeutic composition.

The present invention is also directed to an antimicrobial composition comprising an inhibitory compound that inhibits microbial adherence to or invasion of host mammalian cells, as described in detail herein, and at least one antibiotic effective to treat the microbial pathogen, in a pharmaceutically acceptable carrier. The microbial pathogen is as described above in connection with the therapeutic method of the invention, and can include any microbial pathogen whose infectious process involves adherence to or invasion of host mammalian cells.

Preferably, the antibiotic component of the antimicrobial composition is effective to kill or disable the suspected infectious microbial pathogen. The antibiotic may be an antibacterial, antiviral, antifungal, or antiprotozal compound, and is as described above in connection with the treatment method of the invention. Also as described above, the antimicrobial composition optionally includes at least one of an effective amount of a protein synthesis inhibitor and an effective amount of an inhibitor of a bacterial or host cell enzyme capable of degrading or otherwise inhibiting the activity of the antibiotic. For example, when the antibiotic is penicillin (a β-lactam), a penicillinase (β-lactamase) inhibitor, such as clavulanic acid, can be included in the therapeutic composition. Contemplated formulations of the antimicrobial compositions are as described above with respect to the therapeutic composition used in the treatment method of the invention.

Identification of inhibitory compounds can be accomplished using the principles of rational drug design. The present invention provides a computer-assisted or computer-controlled method that uses, as a starting material, the structure of an integrin, an invasin, an ECM protein, or a previously identified naturally occurring or synthetic inhibitory compound. When based upon the structure of a previously identified inhibitory compound, the computer-assisted or computer-controlled method generates structural analogs of the inhibitory compound which may include, but are not limited to, additions, deletions, substitutions or modifications of substituents, changes in bond saturation levels, addition or deletion of cross-linking and the like. Likewise, when based upon the structure of an integrin, an invasin, an ECM protein, or other biomolecule that mediates internalization by a host mammalian cell of a pathogenic microbe, the computer-assisted or computer-controlled method can be used to generate structural analogs of the biomolecule.

Structural analogs of the starting material identified using the computer-assisted or computer-controlled method as described herein include chemical analogs or homologs of the starting material, such as, for example, an inhibitory compound that contains an amino acid sequences identical to or homologous with an amino acid sequence present in the starting material. Additionally, the term "structural analog" is to be broadly understood to include steric or

electronic analogs of the starting material which may be chemically distant from the starting material but related in other ways. An example of a structural analog considered chemically distant from the starting material from which it is derived is a synthetic organic compound derived from, for example, a peptide ligand starting material that binds to an integrin. The organic compound so derived can have one or more features such as a three-dimensional, space-filling conformation, electronic character, hydrophobicity, hydrophilicity, or the like, in common with or similar to that of the bound peptide ligand starting material, and as a result may competitively inhibit binding of the peptide to the integrin, thereby inhibiting adherence to or invasion of a host mammalian cell by a pathogenic microbe and constituting an inhibitory compound according to the invention.

Where the starting material is a biomolecule involved in microbial internalization by a host cell, the computer-assisted or computer-controlled method for identifying inhibitory compounds can, additionally, be used to design compounds that are not necessarily structural analogs of the starting material, but that potentially bind to the biomolecule so as to block or competitively inhibit one or more intermolecular interactions involved in internalization of the pathogenic microbe by the host cell.

Candidate inhibitory compounds that interfere with interactions between ECM proteins and a host cell integrin or microbial invasin can be initially evaluated *in vitro* by utilizing an enzyme-linked immunosorbant assay (ELISA) using the purified mammalian or microbial receptor protein, for example a mammalian $\alpha 5\beta 1$ integrin or a bacterial M protein, and an ECM protein known to bind to the receptor, as described for example in Example IV. Preferably, the ELISA employs purified ECM protein such as fibronectin, laminin, fibrinogen, and vitronectin. Compounds worthy of further evaluation are those that compete with the binding of the ECM protein to the mammalian or microbial receptor.

Compounds can be further evaluated for their utility as inhibitory compounds in the invention by performing *in vitro* cellular adherence or invasion studies, as exemplified in Examples I, III and IV, to determine whether the candidate compound inhibits adherence and/or invasion of a mammalian cell by an invasive microbe. Essentially, a host cell culture, preferably a culture of

mammalian epithelial or endothelial cells, is exposed to a microbial pathogen in the presence of at least one factor, such as an ECM protein, that stimulates adherence or invasion of the microbial pathogen to the host cell, and a candidate inhibitory compound, for a time effective to allow invasion of or adherence to the host cell by the microbial pathogen. Plasma or serum, such as fetal calf (bovine) serum, can be supplied as the source of the stimulatory factor. The cell culture then is assayed to determine the quantity of internalized or adhered microbial pathogens, and the results are compared to results from a control assay wherein a culture of mammalian epithelial or endothelial cells is exposed to the microbial pathogen in the presence of the stimulatory factor but in the absence of the candidate inhibitory compound, for an equivalent time period. In a preferred embodiment, the screening method of the invention identifies inhibitory compounds that compete with the binding of fibronectin to a fibronectin receptor such as an $\alpha 5\beta 1$ integrin.

The candidate compound can be further evaluated for its utility as an inhibitory compound in the treatment methods and therapeutic compositions of the invention by determining whether, by preventing internalization of the invasive microbe, it increases the effectiveness of an antibiotic against the microbe (see Example VII).

The invention also provides a screening assay that can be used to screen and identify inhibitory compounds from a group of candidate compounds. The assay evaluates competitive inhibition of microbial adherence to and internalization into mammalian cells and can conveniently be performed in an automated system, as by using standard 96 well plates. The mammalian cells used in the screening assay can be either phagocytic or nonphagocytic cells. A monolayer of mammalian cells, preferably epithelial cells or endothelial cells, is preincubated with a candidate compound to be tested for inhibitory activity. A stimulatory factor, such as an ECM protein, that mediates in internalization of the microbe by the host cell, can optionally be provided. Conveniently, the stimulatory factor can be provided in the form of serum (e.g. fetal calf serum) or as a purified ECM protein. The cell monolayer is contacted with a microbe comprising a detectable label, wherein the microbe is capable of internalization into the mammalian cell in the absence of an inhibitory compound. The

detectable label can be located on the surface of the microbe, in the cell wall or membrane, or in an intracellular location within the microbe. Preferably the detectable label is one that can be detected regardless of whether the microbe comprising the detectable label is located outside or inside the mammalian cell, more preferably, the detectable label is a fluorescent label such as a fluorescein-containing compound, e.g. FITC. Other detectable labels that are suitable for use in the screening assay include radiolabels and reporter genes, such as genes encoding for green fluorescent protein (GFP) and β-galactosidase. Instead of using a preincubation step, the candidate compound and the microbe can, alternatively, be supplied at the same time.

Adherence and, optionally, internalization of the detectably-labeled microbe into the mammalian cell are evaluated substantially as described in D. Drevets et al., *J. Immunol. Meth.*, **142** (1), 31-38 (1991), which is incorporated by reference in its entirety. After an incubation period sufficient to permit adherence to and/or internalization of the mammalian cells by the detectably-labeled microbe, typically about two hours, the monolayer is washed to remove any unbound and uninternalized microbe and excess candidate inhibitor. The washed monolayer is then evaluated for the presence of any detectable label. The presence of a detectable label in the monolayer after washing indicates that the microbe is adhering to or has been internalized by the host cells. Absence of the detectable label indicates that the candidate compound has prevented adherence and internalization. The amount of detectable label in the monolayer can be quantified, if desired, to determine the relative effectiveness of the candidate inhibitory compound.

Where the detectable label is a fluorescent label, a further step in the assay can include distinguishing microbial internalization from microbial adherence to host cells by contacting the washed host cell monolayer with an effective amount of an agent that quenches fluorescence originating from extracellular, i.e., adhered, fluorescently labeled microbes. The fluorescence quenching agent used is one that is not internalized by the host cell. Preferably, the fluorescence quenching agent is ethidium bromide. Trypan blue (J. Hed, *Meth. Enzmol.*, **132**, 198-204 (1986)) can also be used to selectively quench extracellular fluorescence. Any fluorescence in the host cell monolayer that

remains after extracellular fluorescence quenching is due to internalization of the fluorescently labeled microbe. The absence of fluorescence indicates that the candidate compound has prevented internalization of the invasive microbe. If desired, fluorescence emission from the monolayers that remains after contact with the fluorescence quenching agent can be quantified to determine the effectiveness of the candidate inhibitory compound.

EXAMPLES

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

Example I. Fetal Calf Serum, Fibronectin, and Laminin Stimulate Intracellular Invasion by M1+ *S. pyogenes*

MATERIALS AND METHODS

Bacterial strains, plasmids and cell media. *S. pyogenes* strain 90-226 is a serotype M1 strain cultured from the blood of a septic patient. This strain was obtained from the WHO Center for Reference and Research on Streptococci at the University of Minnesota (Minneapolis, Minnesota). Strain 90-226 *emm*1::Km was constructed by insertional inactivation of *emm*1 with the *aph*A-3 (kanamycin resistance) gene. *E. coli* DH11S (Life Technologies Inc., Gaithersburg, MD) served as the host for cloning experiments and plasmid maintenance. The protease-deficient *E. coli* strain, BL21, (Navogen Inc., Madison, WI) was used for the expression of the M42-382 fragment of M1 protein encoded by plasmid pM42-382.

Streptococci were grown in Todd-Hewitt broth supplemented with 2% neopeptone (THNB, Difco Laboratories, Detroit, MI). Stock cultures were maintained in 15% glycerol, 85% THNB at -80°C. Solid media for streptococci

were Todd-Hewitt broth or sheep blood agar. *E. coli* was grown in Luria-Bertani broth. Solid media contained 1.5% agar. The plasmid vectors pSport1 and pCYB4 were obtained from Life Technologies and New England Biolabs (Beverly, MA), respectively. Plasmid pMYB129, encoding an *E. coli* maltose-binding-intein fusion protein, was from New England Biolabs. Construction of plasmids pemm1 and pM42-382 is described below.

Plasma proteins and antibodies. Human fibrinogen (Fg) was obtained from Chromogenix Corp., Molndal, Sweden. Human plasma fibronectin (Fn) was obtained from Sigma Chemical Co. and Life Technologies Inc. Mouse laminin-1 (Lm) and human placental laminin (HLm) were purchased from Life Technologies Inc. Human serum albumin and human serum vitronectin were purchased from Sigma Chemical Co, St. Louis, MO. Fibronectin, laminin, albumin and vitronectin were dissolved in RPMI (Life Technologies, Gaithersburg, MD), filter sterilized and stored at 5°C.

Monoclonal antibodies (mABs) against integrins β1 and α5β1 were purchased from Life Technologies Inc. and Chemicon International Inc, Temecula, CA, respectively. Other mAbs recognizing integrin subunits were generously provided by the following University of Minnesota researchers: anti-integrin α2 and α3, Dr. P. Southern; Anti-integrin α5 and α6, Dr. J. McCarthy; anti-integrin β2, Dr. Y. Schimizu; and anti- integrin β4, Dr. A. Skubitz. Sheep anti-human fibronectin was purchased from ICN Pharmaceuticals, Costa Mesa, CA. Alkaline phosphatase conjugated to mouse, anti-sheep IgG was from Sigma Chemical Co.

Cell cultures. Working cultures of streptococci were grown by streaking frozen stock cultures onto sheep blood agar plates. Colonies from the blood agar plates were inoculated into THNB and grown for 6 - 8 hours at 37°C. The THNB-grown cultures were diluted 1:100 into fresh THNB and incubated at 37°C for 14 hours. The bacterial cells were harvested by centrifugation at 600 x-g for 10 minutes at room temperature. The resulting bacterial pellets were suspended in 1 volume of Hanks balanced salt solution (HBSS; Life Technologies, Gaithersburg, MD) then recentrifuged. The final pellets were

resuspended in HBSS to an OD_{560} of 0.5, then diluted into tissue culture media as described below.

A549 human lung epithelial cells (ATCC CCL 185) were cultured in RPMI-1640 Medium supplemented with 10% fetal calf serum (Life Technologies, Gaithersburg, MD). Cultures of A549 cells were maintained in medium containing 5 μg/ml penicillin and 100 μg/ml streptomycin (Sigma Chemical Co.).

Epithelial cell invasion and adherence assays. Invasion assays were performed as in LaPenta et al., *Proc. Nat'l Acad. Sci., U.S.A.*, **91**, 12115-12119 (1994), incorporated herein by reference, with the following modifications. A549 cells were cultured in 24 well plates in RPMI containing 10% FCS without antibiotics. Confluent monolayers were infected with 1-5 x 10^5 bacterial colony forming units (cfu) suspended in RPMI. The assays were conducted in unsupplemented RPMI medium or medium containing RPMI supplemented with FCS, fibronectin or laminin as appropriate. Unless stated otherwise, RPMI-FCS, RPMI-Fg, RPMI-Fn and RPMI-Lm refer to RPMI containing 10% FCS, 25 μg/ml fibrinogen, 10 μg/ml fibronectin or 10 μg/ml mouse laminin, respectively. Plates containing infected monolayers were centrifuged at 200 x g for 5 minutes at room temperature, then incubated for 2 hours at 37°C in 5% CO_2/95% air.

Monolayers were then washed 3 times with 1 ml of HBSS before RPMI-FCS, containing 100 μg/ml gentamicin and 5 μg/ml penicillin, or penicillin alone, was added to each monolayer. Following 2 hours incubation at 37°C, the monolayers were washed 3 times with HBSS, dispersed by the addition of 0.2 ml of 0.25% trypsin, 1 mM EDTA (Life Technologies, Inc.), then lysed by dilution into 0.8 ml of sterile, distilled H_2O. The numbers of bacterial cfu released from the lysed epithelial cells were determined by dilution of lysates in HBSS then plating on Todd-Hewitt agar (Difco).

In some experiments, the total numbers of cfu in wells were determined after the first two hour incubation. Culture media were removed from the monolayers and transferred to 1.5 ml microcentrifuge tubes. Aliquots (0.2 ml) of trypsin-EDTA was then added to the wells and incubated with the monolayers for 10 minutes at room temperature. Aliquots (0.8 ml) of sterile distilled H_20 was added to the trypsinized cells, the solutions were removed from the wells

and transferred to the tubes containing the culture medium. The suspensions were then diluted in HBSS and plated. The total numbers of cfu were used to determine the extent of bacterial growth during the invasion period and to calculate the percentages of adherent cfu.

To measure bacterial adherence, culture media were removed from monolayers at the end of the invasion period and discarded. The monolayers were then washed 3 times with HBSS to remove nonadherent bacteria. Epithelial cells were dispersed and lysed and bacteria were plated as described above. While the numbers of cfu recovered from these wells is reflective of the number of adherent and internalized cfu, for simplicity, we will refer to these bacteria as adherent cfu.

Poly-L-lysine-coated plates were used in invasion experiments that involved the addition of anti-integrin monoclonal antibodies (mAbs). 1 ml of 0.1 mg/ml poly-L-lysine (MW= 30-70 kDa, Sigma Chemical Co.) was added to each well of 24 well tissue culture plates and the plates were incubated for 10 minutes at room temperature. The solution was then aspirated from the wells and each well was washed 3 times with 1 ml sterile, distilled water. Plates were dried in a laminar flow hood, prior to inoculation with A549 cells.

DNA techniques. Plasmid DNA was isolated from *E. coli* strains (DH11S) using the alkaline lysis method. *S. pyogenes* chromosomal DNA was isolated as described (M. Harbaugh et al., *Molec. Microbiol.*, 8, 981-991 (1993). DNA sequencing was performed with reagents purchased from United States Biochemical. Polymerase chain reactions (PCR) were performed using standard procedures (see, e.g., J. Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)).

The *emm*1 gene of strain 90-226 was amplified via PCR using primers complimentary to the conserved 5' portion of M genes (GGGGGGGGATCCATAAGGAGCATAAAAATGGCT) and nucleotides 55-30 of the *sic* gene (AAGAAAGGATCCAAGGGATGTAAATAGTAGTGT) (P. Akesson et al., *J. Biol. Chem.*, 271, 1081-1088 (1996)). *Bam*HI restriction sites were added to the 5' ends of the primers to facilitate cloning of the fragment. The amplified DNA fragment was digested with *Bam*HI, ligated with *Bam*HI-

digested pSportI, and transformed into *E. coli*. One plasmid isolate, designated pemm1, was chosen for further study. Restriction enzyme mapping and DNA sequencing were performed to verify that the plasmid carried *emm*1.

For expression of M1 fragments in *E. coli*, a portion of the *emm*1 gene was PCR-amplified using pemm1 as template. The oligonucleotide primers used were complimentary to nucleotides (nt) 154-174 (GCGATGTCATGAACGGTGATGGTAATCCTAGG) and 1177-1156 (AGTCCCCCGGGAAGTTTTGCTTGTAGTTCAGC) of *emm*1 (M. Harbaugh et al., *Molec. Microbiol.* 8, 981-991 (1993)). The amplified fragment encodes the first 341 amino acid residues of the mature M1 protein (residues 42 to 382). The DNA fragment was purified, cut with *Bsp*HI and *Sma*I, then ligated into *Nco*I-*Sma*I digested pCYB4, to construct the plasmid, pM42-382. Cloning of the *emm*1 fragment into pCYB4 resulted in the construction of a hybrid gene. where the M1 coding region is fused, at its 3' terminus, to the N-terminal coding region of an intein-chitin binding protein (IMPACT 1: One-step protein purification system, technical manual, New England Biolabs, Inc., Beverly MA (1996)). The fusion protein has a predicted molecular weight (MW) of 94.5 kDa. *E. coli* transformed with pM42-382 can be induced to express the fusion protein which can then be purified by chromatography of bacterial sonicates on a chitin affinity column (New England Biolabs). The addition of dithiothreitol to the column promotes intein-mediated, autocleavage of the fusion protein, releasing the M42-382 fragment from the affinity matrix. The purified M42-382 fragment (MW= 39.5 kDa) is predicted to possess an N-terminal, formyl-methionine and a C-terminal glycine residue not present in native M1.

General protein techniques. Concentrations of protein solutions were determined according to the Bradford method using bovine serum albumin as standard. SDS/PAGE was performed according to Laemmli. For Western blotting, proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) using a trans-blot cell apparatus (Biorad). Membranes were blocked with 0.25% gelatin, washed briefly in TBST (20 mM Tris, pH7.5, 0.5 M NaCl, 0.05% tween 20), then incubated with anti-Fn Ab in TBST. Membranes were then incubated with alkaline phosphatase conjugated to mouse, anti-sheep IgG, washed and finally developed using BCIP/NBT phosphatase

detection reagents (Life Technologies Inc.). Detection of fibronectin binding to immobilized M42-382 was performed similarly, except membranes were blocked with 5% nonfat dry milk and incubated with 25 μg/ml fibronectin prior to incubation with Abs.

Protein purifications. Fibronectin and fibrinogen were purified from commercially available fibrinogen as follows. 250 mg of lyophilized protein was suspended in 50 ml Hanks balanced salt solution (HBSS; Life Technologies) containing 1 mM PMSF and filtered through a 0.22 um cellulose acetate filter to remove insoluble material. The protein was loaded onto a 1.5 by 5.7 cm gelatin-Sepharose (Pharmacia) column. Chromotography was performed at room temperature. The effluent, containing Fn-depleted fibrinogen, was collected and aliquots were stored at -20°C. No fibronectin was detected in Western blot analysis of the repurified fibrinogen, using anti-Fn Ab. To recover fibronectin, the column was washed with 50 ml HBSS-PMSF, followed by 0.05 M sodium acetate, 1 M sodium bromide. 1.5 ml fractions were collected and protein-containing fractions were pooled, then dialyzed against HBSS. Aliquots of the purified fibronectin were stored at –20°C.

For purification of the M42-382 fragment, BL21/pM42-382 was grown in LB plus ampicillin, at 37°C, to an optical density at 600 nm (OD_{600}) of 0.2 and then at room temperature to an OD_{600} of 0.5. At that time, IPTG was added to 1 mM and the culture was incubated at 15°C for 16-18 hours. Cells were harvested by centrifugation and suspended in 50 ml of column buffer (20 mM Tris, pH8.0, 0.5 M NaCl, 0.1 M EDTA, 0.1% triton x-100) containing 1 μg/ml DNaseI and 1 mM PMSF. All subsequent steps were performed at 4°C. Bacterial cells were disrupted by passage through a French press at 8000 psi. Lysates were then clarified by centrifugation and the resulting supernatants were loaded onto a 1.5 by 5 cm chitin-affinity column (New England Biolabs). The column was washed with 100 ml of column buffer, followed by 15 ml of cleavage buffer (20 mM Tris, pH8, 50 mM NaCl, 0.1 mM EDTA) containing 30 mM dithiothreitol. The column was incubated with cleavage buffer for 16 hours at 4°C. The M1 fragment was then eluted by the addition of 15 ml cleavage buffer. The eluted protein was dialyzed against 50 mM ammonium acetate, pH 6.9, then

concentrated by lyophilization. Lyophilized protein was suspended in PBS to 1 μg/ml, aliquoted and stored at -80°C.

Fibronectin binding assay. Fibronectin, suspended in 0.1 M sodium phosphate, 0.15 M NaCl, pH 6.5, was labeled with ^{125}I (Amersham) using Iodobeads iodination reagent (Pierce Chemical Co.) to a specific activity of approximately 10^6 cpm/μg protein. Iodinated protein was separated from free label by chromatography on cross-linked dextran columns. Fractions were collected and those containing ^{125}I-Fn were pooled, aliqouted and stored at -20°C.

Assay of fibronectin binding to streptococci was performed as described (E. Hanski et al., *Proc. Natl. Acad. Sci., U.S.A.*, **89**, 6172-6176 (1992)). Briefly, overnight cultures of streptococci were harvested by centrifugation. washed twice with 1 volume PBS, pH7.4, and finally suspended in PBSAT (PBS containing 0.02% sodium azide and 1% tween 20) to an OD_{600} of 0.5. 100 μl portions of the cell suspensions were diluted into 800 μl PBSAT. 1-100 μl of ^{125}I-Fn was then added to duplicate tubes of cell suspension and the final reaction volumes were adjusted to 1 ml using PBS. Bacteria and labeled protein were then incubated at room temperature, with end-over-end rotation, for 2 hours. 50 μl of *E. coli* DH11S, suspended at an OD_{600} of 10 in PBSAT, was then added to each tube. Tubes were centrifuged at 14,000 x g for 10 minutes at room temperature. Bacterial pellets were washed once with 1 volume PBSAT and the amount of radioactivity associated with the pellets was determined. Nonspecific binding was determined by adding the same amount of labeled fibronectin to tubes containing no streptococci. These reactions were incubated and bound counts were determined, as described above. Radioactive counts recovered from these reactions were subtracted from those obtained from tubes containing streptococci.

RESULTS

Effect of sera and purified plasma proteins on adherence and internalization of M1+ and M1- streptococci. Isogenic M1$^+$ and M1$^-$ strains were tested for their responses to FCS, fibronectin or laminin addition, with

regard to invasion of A549 cells. The results are shown in Fig. 2. $M1^+$ and $M1^-$ bacteria were suspended in RPMI medium containing no agonist (designated "None" in the graph) or in RPMI medium containing the indicated agonist, just prior to infection of monolayers. Fig. 2a shows intracellular invasion, wherein percent invasion was calculated as (internalized CFU/CFU in the inoculum) times 100. Fig. 2b shows streptococcal adherence to A549 cells, wherein percent adherence was expressed as the percentage of total CFU that remained associated with monolayers after 3 successive washings with buffer. Data are the means ± standard errors of the means, from 6-9 infected monolayers (2 or 3 experiments, each performed in triplicate).

Intracellular invasion of A549 epithelial cells by *S. pyogenes* strain 90-226 was found to be highly dependent upon the presence of FCS with, typically, less then 1% recovery of the bacterial inoculum, following incubation with antibiotics. The addition of 10% FCS stimulated invasion greater than 50-fold. In control experiments, FCS was found to have no effect upon the sensitivity of strain 90-226 to gentamicin and penicillin. These results suggested that FCS contained at least one factor which directly stimulated intracellular invasion. Indeed, intracellular invasion of A549 epithelial cells by *S. pyogenes* strain 90-226 was shown to be stimulated by the addition of the purified plasma protein fibronectin (Fn) and the extracellular matrix (ECM) protein laminin (Lm).

To determine if expression of M1 protein is required for the stimulation of invasion by these factors, invasion and adherence assays were performed with strain 90-226 ($M1^+$) and 90-226 *emm*1::Km ($M1^-$). It was found that invasion by either strain was very inefficient in the absence of an agonist, with less than 1% internalization of the bacterial inoculum (Fig. 2a). The addition of FBS, fibronectin or mouse laminin stimulated internalization of $M1^+$ bacteria by 35, 70 and 50-fold, respectively. In contrast, invasion by the $M1^-$ strain was unaffected by FBS or fibronectin addition and was stimulated only 2-fold by laminin.

$M1^+$ and $M1^-$ streptococci both adhere well to A549 cells in the absence of an invasion agonist. Typically, 20-30% of the wild type and 25-35% of the $M1^-$ CFU remain associated with epithelial cells after washing of the infected monolayers (Fig. 2b). FBS, fibronectin or laminin stimulate adherence of the $M1^+$ strain approximately 2-fold. Fibronectin- and laminin-stimulated adherence

is apparently M1-dependent, as adherence of the M1⁻ mutant was not increased by these factors. FBS was found to increase adherence of M1⁻ bacteria by approximately 30%. The slightly higher adherence of the M1⁻ mutant, in the absence of agonists, may result from upregulation, or increased cell-surface exposure, of another adhesion in the mutant strain.

These results demonstrate that strain 90-226 possesses a mechanism for invasion of epithelial cells that involves M1 expression together with the presence of either fibronectin or laminin.

Inhibition of invasion by fibronectin antiserum. We previously reported that human fibrinogen could activate intracellular invasion by strain 90-226, whereas fibronectin could not (D. Cue et al., *Infect. Immun.* 65, 2759-2764 (1997)). We have since determined that the fibrinogen preparation used in earlier experiments contained approximately 1.6% fibronectin. The fibrinogen and fibronectin in this mixture were separated by chromatography on gelatin-Sepharose and the recovered proteins were tested for invasion stimulation activity. The fibronectin-depleted fibrinogen failed to stimulate invasion, while the fibrinogen-depleted fibronectin was an active agonist. It is not clear why the fibronectin preparation used in the previous study (D. Cue et al., supra) failed to promote invasion. A second preparation of fibronectin, obtained from the same supplier (Sigma Chemical Co., St. Louis, MO) was also inactive. Bovine and human plasma fibronectin, obtained from a different commercial source (Life Technologies Inc.), had activities comparable to the fibronectin purified from the fibrinogen/fibronectin mixture (Chromogenix Corp, Molndal, Sweden).

In light of these results, it seemed plausible that our laminin preparations could contain fibronectin, or vice versa, and that only one of these proteins facilitated invasion. It was also possible that all of the active protein preparations may have contained a common contaminant that was the true invasion agonist. Staining of SDS/PAGE gels, however, did not reveal a contaminating factor that was present exclusively in active protein preparations. Dialysis of active protein preparations did not result in any appreciable losses in activity, thus it is unlikely that agonistic activity is due to a low-molecular-weight, contaminating factor. Moreover, anti-fibronectin antibody (anti-Fn Ab)

did not react with an active preparation of human laminin (HLm) or with any peptide found exclusively in active preparations.

As shown in Fig. 3, anti-Fn Ab was found to inhibit internalization of streptococci when the agonist was FBS, fibronectin or the fibrinogen/fibronectin mixture. Sheep anti-human Fn Ab was added to RPMI medium containing the indicated agonist. M1$^+$ bacteria were added to these media and to identical media to which no antibody was added. The bacterial suspensions were then added to A549 monolayers. Thereafter, our standard invasion procedure was followed. Y axis values are numbers of CFU recovered from mAb containing wells, divided by the numbers of CFU recovered from control wells, containing no mAb, multiplied by 100. Data are means and ranges from assays performed in triplicate. Interestingly, the same antiserum did not appreciably affect HLm-mediated invasion (Fig. 3) nor did it adversely affect bacterial growth. These results are all consistent with fibronectin and laminin being distinct, bona fide invasion agonists.

Sequencing of *emm*1. The *emm*1 gene of strain 90-226 was amplified by PCR using oligonucleotides complementary to the M protein signal sequence (M. Harbaugh et al., *Molec. Microbiol.*, **8**, 981-991 (1993); A Podbielski et al.; *Med. Microbiol. Immunol.*, **180**, 213-227 (1991)) and to a sequence in the 5' coding region of *sic* (P. Akesson et al, *J. Biol. Chem.*, **271**, 1081-1088 (1996)). The PCR fragment was cloned into pSport1 and transformed into *E. coli*. One plasmid isolate, pemm1, was chosen for sequencing of the cloned fragment. The entire 1724 base pair (bp) insert of pemm1 was sequenced and found to contain an open reading frame of 1452 bps, predicted to encode a 484 amino acid protein. The 1452 bp segment was 99.4% homologous to the *emm*1.0 gene of *S. pyogenes* AP1 (P. Akesson et al., *Biochem. J.*, **300**, 877-886 (1994)). Out of the 8 nucleotide substitutions found, only 1 is predicted to affect the amino acid sequence of M1 protein. Amino acid residue 366 is predicted to be glutamic acid in the AP1 protein and glycine in the strain 90-226 peptide. This result is consistent with the report of Musser et al. (*Infect. Immun.*, **63**, 994-1003 (1995)) that the globally-disseminated, M1 subclone, responsible for the majority of contemporary, invasive streptococcal infections, carries the *emm*1.0 allele. The last 54 bps of the sequenced fragment were 100% homologous to the N-terminal

coding region of *sic*. A 190 bp sequence was found between the *emm*1 stop codon and the *sic* start codon. The *sph* gene, found between *emm*1 and *sic* in some M1 strains, is apparently absent from this segment of the strain 90-226 chromosome. Thus, the *emm*1 and *sic* genes of strain 90-226 are closely linked and separated by 190 bps of DNA.

Fibronectin binding by M1 protein. A number of *S. pyogenes* isolates express a cell-surface, fibronectin-binding protein, protein F, that mediates bacterial adherence to host cells. Protein F has been shown to foster adherence by binding soluble fibronectin which, in turn, binds to the extracellular matrix of host tissues. Fibronectin and laminin could promote intracellular invasion by $M1^+$ streptococci via a similar mechanism, although we know of no studies demonstrating binding of either agonist by M1 protein.

As a partial test of this mechanism, we first compared binding of ^{125}I-Fn to $M1^+$ and $M1^-$ bacteria. The results of a representative experiment are presented in Fig. 4. Constant numbers of bacterial cells were suspended in PBST, mixed with varying amounts of ^{125}I-Fn, and rotated, end-over-end, for 2 hours, at room temperature. Bacterial cells were harvested by centrifugation and the amount of radioactivity associated with the bacterial pellets was determined. The values plotted are the average counts recovered from duplicate tubes. Nonspecific binding was determined by adding the same amounts of labeled protein to tubes containing no bacteria. Counts recovered from these tubes were subtracted from the values plotted in the figure.

$M1^+$ bacteria consistently bound fibronectin with a greater affinity than did $M1^-$ cells. Unlabelled fibronectin was found to effectively compete with labeled fibronectin for binding to wild-type cells, indicating that binding was saturable. The experiment was repeated with a different preparation of ^{125}I-Fn and yielded similar results. Overall, fibronectin binding was reduced 60-90% for the $M1^-$ mutant.

While these results were consistent with direct binding of M1 to fibronectin, other explanations for these results were possible. Therefore, we tested whether purified M1 could bind fibronectin. To accomplish this, a DNA fragment coding for amino acids residues 42 to 382 of M1 was cloned into the *E. coli* expression vector pCYB4. The 343 amino acid peptide encoded by this

fragment corresponds to the extracellular portion of M1 plus 2 amino acids encoded by the vector. The recombinant protein (M42-382) was expressed in *E. coli* and purified as described above. Western blot analysis of M42-382 indicated that the purified protein reacted with M1 antiserum and could bind human fibrinogen. Thus, the recombinant protein possessed the properties predicted of M1 protein.

To test for fibronectin binding to M42-382, the protein was electrophoresed on SDS/PAGE gels then transferred to nitrocellulose membranes. As controls, *E. coli* maltose binding protein, purified from a strain carrying a derivative of pCYB4, and/or ovalbumin were run in separate lanes of the gels. Specifically, purified M42-382 protein and *E. coli* maltose binding protein were each electrophoresed through 10% acrylamide/SDS gels and transferred to nitrocellulose membrane. The membrane was blocked, then sliced to remove the portion of the membrane containing the maltose binding protein sample. The portion of the membrane containing the M42-382 protein samples was successively incubated with 25 ug/ml fibronectin in Tris-buffered saline (TBS), sheep anti-human fibronectin antibody, alkaline phosphatase conjugated to mouse anti-sheep IgG, and finally a chromogenic alkaline phosphatase substrate. Fibronectin binding by M1 protein was readily detected in this assay. As a control for binding of antibodies directly to M1 protein, duplicate membranes were incubated with the primary and secondary antibodies, without prior incubation with fibronectin. Either no signal or a weak signal was obtained from this control. We conclude that M1 protein can directly bind fibronectin and that this interaction accounts for most of the fibronectin binding by strain 90-226.

Effects of anti-integrin monoclonal antibodies on intracellular invasion efficiency. Since invasion by strain 90-226 is dependent upon binding of fibronectin or laminin, the involvement of host integrin(s) in internalization of streptococci was investigated. Several monoclonal antibodies (mAbs) were assayed for inhibition of invasion by M1$^+$ bacteria. In initial experiments, varying amounts of mAbs, directed against integrins $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$, $\beta 1$, $\beta 2$, and $\beta 4$, were incubated with monolayers, prior to infection with bacteria. These invasions were performed in the presence of added fibronectin.

Monoclonal antibodies directed against the integrin α5 and β1 subunits were found to inhibit fibronectin-mediated invasion, the remaining mAbs had slight to no inhibitory effects. Control experiments were performed to verify that the inhibitory mAbs did not adversely affect bacterial viability or adherence of A549 cells to the substratum. To verify the results of the screening assays, all of the mAbs were retested, using a constant concentration of each antibody (Fig. 5). Specifically, monoclonal antibodies recognizing the indicated integrin subunits were diluted in RPMI medium containing fibronectin and incubated with A549 monolayers, for 30 minutes, prior to addition of bacteria. Thereafter, the standard assay procedure was followed. Purified mAbs against integrins α5 and β1 were diluted to 0.35 and 0.5 μg per ml, respectively. Other mAbs were diluted 1:50, final concentrations of antibodies varied from 4.8 to 38 μg per ml. Y axis values are numbers of CFU recovered from mAb-containing wells, divided by the numbers of CFU recovered from control wells, containing no mAb, multiplied by 100. Data are means and ranges from assays performed in triplicate.

The results shown in Fig. 5 confirmed our previous observations. Since the two inhibitory mAbs should both react with the epithelial cell fibronectin receptor, integrin α5β1, we obtained a mAb that specifically recognizes this receptor (S. Caixia et al., *Cell. Immunol.*, **138**, 216-228 (1991)) and tested it for invasion inhibition. This antibody also inhibited fibronectin-mediated invasion, suggesting that fibronectin stimulates internalization of M1$^+$ streptococci by facilitating bacterial interaction with integrin α5β1.

The potential of anti-integrin β1 and α5β1 mAbs to inhibit invasion promoted by agonists other than fibronectin was also evaluated (Fig. 6). Monoclonal antibodies directed against integrin β1 or integrin α5β1 were added to RPMI medium containing the indicated agonists. M1$^+$ bacteria were added to these media and to identical media to which monoclonal antibody was not added. The bacterial suspensions were then inoculated onto A549 monolayers. Thereafter, the standard assay procedure was followed. Y axis values are the numbers of internalized CFU recovered from control wells, containing no antibody, were assigned a value of 100%. Results from Ab-containing wells are expressed as percentages of the controls. Data are mean percentages and ranges,

from a representative experiment, in which each assay was performed in triplicate.

As shown in Fig. 6, anti-integrin β1 mAb inhibited fibronectin, laminin and FBS-mediated invasion by 91%, 98% and 86%, respectively. Invasion in the presence of the GRGDTP peptide (see Example II) was also inhibited by 23% but this inhibition appeared not to be specific for the peptide-promoted invasion pathway, since a comparable level of inhibition (21.5%) was observed when no agonist was present.

The anti-integrin α5β1 mAb inhibited fibronectin-mediated invasion by 94% and FBS-mediated invasion by 80% (Fig. 6b). Laminin-mediated invasion did not seem to be specifically inhibited by this Ab since the level of inhibition (32%) was comparable to that observed in the absence of an agonist (26%). The "nonspecific effects" of the mAbs could result from blocking bacterial access to other receptors, cross-reactions with other integrins or, perhaps, the result of physiological effects on the epithelial cells.

A bridging effect, i.e., between a host cell integrin and bacterial M1 protein, may account for fibronectin and laminin-mediated invasion by $M1^+$ streptococci. As demonstrated above, the *emm*1::Km mutation reduces fibronectin binding by strain 90-226 by 60-90%. Additionally, purified M1 protein is capable of binding fibronectin. These results are consistent with the proposal that fibronectin functions as a bridging molecule between M1 and integrin α5β1. We are unaware of any previous reports of fibronectin binding by M1, although M3 protein is known to bind fibronectin (K.-H. Schmidt, *FEMS Immunol. Med. Microbiol.*, 7, 135-144 (1993)) and Frick et al. (*EMBO J.*, **14**, 1674-1679 (1995)) have reported that the streptococcal M-like protein, protein H, binds to type III fibronectin repeats.

The fact that antibodies directed against the epithelial cell fibronectin receptor, α5β1, specifically abrogates fibronectin-mediated invasion suggests that fibronectin targets bacterial binding to at least this integrin. Interaction with this receptor could result in ligand-mediated endocytosis of bacteria by A549 cells. Laminin likely fosters bacterial interaction with one or more integrins (α1β1, α2β1, α3β1, α6β1, or α7β1) for which laminin is a ligand (R. Hynes, *Cell*, **69**, 11-25 (1992); R. Timpl et al., *Matrix Biol.*, **14**, 275-281 (1994)).

Although monoclonal antibodies against the integrin α2, α3 and α6 subunits were used in this study, these mAbs have, thus far, only been tested for inhibition of fibronectin-mediated invasion.

The mechanism underlying M1-dependent entry of streptococci into cultured cells seems to most closely resemble that underlying uptake of *Y. pseudotuberculosis*. *Yersinia* also encodes multiple, independent pathways for entry into mammalian cells. One pathway is mediated by invasin, a 108 kDa outer membrane protein capable of binding at least four different β1 chain integrins, including the α5β1 receptor (R. Isberg et al., *Cell*, **60**, 861-871 (1990)). However in *Yersinia*, interaction of invasin with α5β1 is not dependent on fibronectin binding by either receptor. Rather, the bacterial cell surface protein invasin binds directly to the host cell integrin α5β1 with high affinity, and binding can be inhibited by fibronectin or RGD-containing peptides (G. Van Nhieu et al., *J. Biol. Chem.*, **266**, 24367-24375 (1991)). This mechanism is clearly distinct from that used by M1$^+$ streptococci, which is mediated by ligands.

In summary, antibodies directed against fibronectin or integrin α5β1 were both effective at blocking invasion stimulation by fibronectin, however, neither antibody abrogated laminin-mediated invasion. These results suggest that, despite the fact that fibronectin and laminin both utilize M1 protein expression to stimulate invasion, these two agonists apparently target bacteria to distinct, host-cell integrins.

Example II. RGD Peptides Stimulate Intracellular Invasion by Both M1+ and M1- *S. pyogenes*

A number of adhesive proteins, including fibrinogen, contain the short amino acid sequence, RGD. The $\alpha_{IIb}B_3$ integrin, for example, is known to recognize three amino acid sequences within fibrinogen. Two of these (RGDS and RGDF) are RGD-containing sequences within the α chain of fibrinogen. The third sequence is a twelve amino acid sequence (HHLGGAKQAGDV) at the C-terminal end of the γ subunit. The effect of several peptides containing the RGD sequence on invasion by M1+ and M1- *S. pyogenes* was evaluated. The

dodecapeptide, lacking an RGD sequence but nonetheless containing a sequence recognized by $\alpha_{IIb}B_3$ integrin, served as a presumed negative control.

Materials and Methods. The six amino acid peptide (GRGDTP) was purchased from Sigma Chemical Co. Peptides RGD, RGDS and HHLGGAKQAGDV (dodecapeptide), and the cyclic peptide N-acetyl-L-penicillamine-RGDC, were purchased from Bachem California Inc., Torrance, CA. Lyophilized peptides were dissolved in RPMI medium and filter sterilized; aliquots were stored at -80°C. *S. pyogenes* strains 90-226 (M1+) and 90-226 *emm*1::Km (M1-) were

Invasion and adherence assays were conducted as described in Example I. Assays were also performed where bacteria were preincubated with peptide, and the bacterial cells pelleted and washed prior to infection of monolayers.

Results. The six amino acid peptide GRGDTP was found to stimulate invasion by *S. pyogenes* strain 90-226 (M1+) by 5 to 10-fold when RPMI was supplemented with ≥250 μg/ml of peptide. Although peptide addition had its largest effect upon invasion, it also had a modest effect on bacterial adherence (Table 1). The agonistic factors (fibronectin, laminin, FCS and GRGDTP peptide) thus exerted their greatest effect on the internalization of adherent bacteria. The invasion index (internalized cfu/adherent and internalized cfu) of strain 90-226 was increased 20 to 30-fold by the addition of fibrinogen or FCS, whereas the GRGDTP peptide increased the invasion index by approximately 5-fold. Preincubation of bacteria in RPMI-peptide resulted in a 3.2-fold increase in invasion efficiency, relative to invasions by bacteria that had been preincubated with RPMI. The effects of peptide on invasion thus closely paralleled the agonistic effects of fibronectin and FCS on invasion.

Table 1. Invasion steps affected by RGD peptide addition[a]

Invasion medium	% Invasion	% Adherence	Invasion Index
RPMI	0.28 (0.28-0.29)	12.8 (11.0-14.8)	5.0×10^{-3} ($5.0\text{-}6.0 \times 10^{-3}$)
RPMI-peptide[b]	4.04 (3.5-4.9)	19.1 (16.3-21.9)	2.5×10^{-2} ($2.1\text{-}2.9 \times 10^{-2}$)
RPMI-Fibrinogen/ fibronectin	17.0 (8.06-29.7)	16.9 (9.30-31.1)	1.47×10^{-1} ($7.0\text{-}26.0 \times 10^{-2}$)

[a]Percent invasion, =percent adherence and invasion indices were calculated as described in the legend to Table 3.

[b]RPMI supplemented with 250 μg/ml of the 6 amino acid peptide, GRGDTP.

The RGDS peptide and cyclic peptide both stimulated invasion by *S. pyogenes* strain 90-226 (M1+), and the extent of invasion was proportional to peptide concentration. The tripeptide (RGD) and dodecapeptide control failed to stimulate invasion by the M+ strain at any concentration. In order to directly compare the abilities of the peptides to enhance invasion, the extent of bacterial internalization in RPMI containing 0.4 mM of each peptide was compared (Table 2). The data indicate that the cyclic, RGDS and GRGDTP peptides have comparable abilities to stimulate invasion. None of the peptides were found to stimulate growth of strain 90-226.

Table 2. Invasion stimulation by RGD peptides

Peptide[a]	% Invasion[b]
GRGDTP	4.39
	(2.96-4.89)
RGD	1.07
	(0.82-1.36)
RGDS	4.11
	(2.42-5.85)
cyclic[c]	3.06
	(2.23-3.35)
HHLGGAKQAGDV	0.97
	(0.76-1.11)
none	0.91
	(0.89-0.94)
fibrinogen/fibronectin	12.1
	(8.65-12.7)

[a] Strain 90-226 was allowed to invade A549 epithelial cells in RPMI (none), RPMI containing 25 μg/ml fibrinogen/fibronectin or 0.4 mM of the indicated peptide.

[b] (intracellular cfu/cfu in inoculum) X 100. Data are the mean percentages and ranges from experiments performed in triplicate.

[c] acetyl-L-penicillamine-RGDC.

The potential of the same peptides to promote invasion by the M1⁻ derivative of strain 90-226 was also tested. Fig. 7 shows the results. M1⁻ bacteria were suspended in RPMI medium containing no peptide agonists (designated "None" in the histogram) or in RPMI medium containing 0.5 mM of the indicated peptide, then inoculated onto monolayers. Percent invasion was calculated as (internalized CFU/CFU in the inoculum) times 100. The values represented in the graph are the mean percentages from a representative experiment, in which each assay was performed in triplicate.

Peptides of the sequence RGDS and GRGDTP stimulated invasion by 6 and 12-fold, respectively, whereas a cyclic peptide increased invasion by approximately 8-fold. The tripeptide, RGD, had no measurable impact on bacterial internalization. These results are nearly identical to those obtained from experiments with the M1⁺ strain, therefore, M1 expression is not required for RGD peptide-promoted invasion.

As noted in Example I, anti-integrin β1 monoclonal antibodies did not block peptide-promoted invasion, whereas the same monoclonal anbibody inhibited invasion in the presence of fibronectin or laminin. Taken together, these results demonstrate that strain 90-226 possesses at least three distinct mechanisms for invasion of epithelial cells: two mechanisms that are dependent upon M1 expression and, in one case, the presence of fibronectin and in the other case, the presence of laminin; and a second, M1- independent mechanism, that is stimulated, for example, by exposure to RGD-containing peptides.

Example III: Characterization of Selected Integrin Antagonists

Materials. Adenosine 5'-diphosphate (ADP) and other reagents used but not specifically mentioned were obtained from Sigma Chemical Company (St. Louis, MO). Vitronectin (Collaborative Biomedical Products), fibrinogen (Enzyme Res. Lab., Indianapolis, IN or Sigma Chem. Co.), fibronectin (New York Blood Bank or Sigma Chem. Co.) and other matrix proteins such as osteopontin, CS-1 or thrombospondin were either made in house or received as a

gift from our collaborators. All small molecule integrin antagonists were synthesized at DuPont Merck Pharmaceutical Co., Wilmington DE.

A. α5β1 Affinity Assays

i) Purified human α5β1 Receptor-Biotinylated Fibronectin Binding Assay. Purified receptor obtained from human placenta was diluted (1:2000) with coating buffer and coated (100 μL/well) onto Costar (3590) high capacity binding plates overnight at 4°C. The coating solution was discarded and plates were washed once with binding buffer (B/B buffer: 50mM TrisHCl, 100mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 1mM $MnCl_2$, ph 7.4). The wells were then blocked with 200μL B/B buffer containing 1% BSA. After washing once with B/B buffer, 100 μL of biotinylated fibronectin (2nM- 1:5, 125 in the binding buffer) was added plus 11 μL of either inhibitor or B/B buffer containing 1.0% BSA to each well and incubated for 1 hour at room temperature. The plate was washed twice with B/B buffer and then incubated for 1 hr at room temperature with 100 μL alkaline phosphatase. Color was developed and measured at room temperature for approximately 45 minutes as above.

ii) α5β1 Affinity: α5β1-Mediated Cell Adhesion Assay. Fibronectin was plated onto 96-well plates (Costar 3590, high binding, flat bottom) at 50 μg/ml overnight at 4°C. The protein solution was removed from the wells and washed twice with PBS containing protease inhibitor. The non-specific binding was blocked by adding 150 μL of PBS plus 1% BSA for 2 hours at room temperature or overnight. On the day of experiment, Jurkat cells were obtained and centrifuged for 10 minutes at 1,200 rpm. Jurkat cells are a T-cell cell line with high concentrations of α5β1 integrins. After centrifugation (at 300 rpm x 2 for 10 minutes) Jurkat cells in ~2 ml of standard RPMI medium plus ~20-30 ml medium were then resuspended in ~2 ml medium and counted in coulter counter. Cells were labeled by adding 2 μM of calcein-AM per $3\text{-}5x10^6$ cells/ml at 37°C for 30 minutes with occasional shaking. Test agents at different concentrations were incubated for 30 minutes, 37°C, in a dark incubator. Labeled Jurkat ~$2x10^5$ cells were added into each well and incubated at room temperature for 1 hour on a rotator. After incubation, wells were washed twice

with RPMI, then 150 μL RPMI was added back into the wells and fluorescence read in Cytofluor 2300 within an hour, Ex:485 nm (filter B) EM: 530 nm (filter B).

B. αvβ3-Mediated Cellular Adhesion Assays

i) Endothelial Cell-Fibrinogen (αvβ3-mediated) Adhesion Assay: The αvβ3-selectivity of HUVEC (human umbilical venous endothelical cell) adhesion to fibrinogen was characterized with an αvβ3 monoclonal antibody, LM609. The assay is as previously described (S. Mousa et al., *Coronary Artery Disease*, 7 (10): 767-774, 1996) in which a Costar 3590 plate was coated with 100 μl of fibrinogen (25 μg per well) over night at 4°C. Following overnight coating, each well was washed twice with 200 μL of PBS. Non-specific binding was blocked by adding 200 μL of PBS + 5.0% BSA per well for 1 hour at room temperature. Resuspended HUVECs or endothelial or smooth muscle cells (~1 x 10^6 cells/mL) obtained from various species in MCDB-131 per method standard media were used when confluent, passages 3-4. Cells were labeled with 2 μM Calcein-AM (Molecular Probes, #C3100, 50 μg per vial) for 30 minutes at 37°C in a humidified incubator. The vial containing labeled HUVECs was wrapped in foil to prevent photo bleaching of the dye. Following calcein labeling, cells were washed twice with ~40 mL of MCDB-131 media, centrifuged 15 minutes at 1000 rpm and then resuspended; cell counts were adjusted to $1x10^6$/mL. Cells were preincubated with 150 μL of test compounds or media for 15 minutes at room temperature and added to the assay plate and incubated for another 60 minutes at room temperature. The plate was covered with foil to prevent photo bleaching of the dye from labeled HUVECs. Following the drug/cell and matrix interaction, media was removed from the wells and washed twice with 200 μL of MCDB-131. Following washing, 100 μL of MCDB-131 media was added to each assay well and the fluorescence was read on a Cytofluor 2300 at sensitivity 2 or 3, Ex=485 nm and EM=530nm.

ii) 293/β3 - Fibrinogen Adhesion. ELISA plate wells were coated with fibrinogen 1-4 nights before the assay at 25 μg/well and stored at 4 C until use. The day of the assay, the fibrinogen was removed, wells were washed twice with

PBS without cations, and the wells were blocked with 5% BSA made in PBS without cations for 2 hours. 293 β3 Z cells at 30-70% confluency were harvested and brought up to 1 x 10^6 cells/ml in MCDB131+25nM HEPES, pH 7.5 (AB). Compounds were made at 40 X the desired assay concentrations in AB + .2% DMSO and non specific adhesion was measured by assaying 200 nM SM256. On a 96 well polypropylene plate, 65 µl of AB was added followed by 5 µl of compound solution. Then 130 µl of cells was added and incubated for 15 minutes at 37°C, 5% CO2 for 15 minutes. After the BSA was removed from the adhesion assay plate, 150 µl of the preincubation mix was added to each well and incubated for 1 hour at 37°C, 5% CO2. After the incubation, the non adhered cells were removed using an Impact2 matrix pipette at the second fastest speed (fill 25 µl, mix 50 µl, fill 200 µl). 200 µl of PBS with cations was added directly on top of cells using an Impact 2 matrix at its second slowest speed setting. Cells were lysed with 100 mM potassium phosphate, .2% Triton X-100, 1mM DTT, pH 7.8 solution, and 5 µl was assayed for β-galactosidase using a GalactoLight Plust Assay (Tropix) following a modification of the manufacturer's suggested protocol (50 µl Chemiluminescent Substrate, 1 hour incubation, 50 µl of Accelerator II). Luminescence values were converted to β-galactosidase using a standard curve, non specific was subtracted, and % inhibition was calculated for each compound concentration. Finally, IC50s were computed using Kaleidagraph's curve fitting capabilities (%I = ([conc]*100/([conc]+IC50).

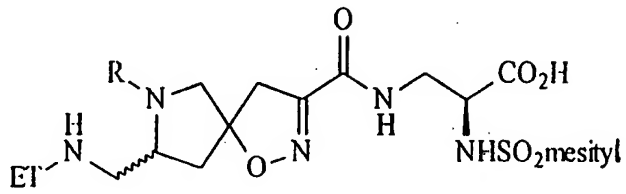
C. Antiplatelet $\alpha_{IIb}\beta_3$ Efficacy.Light Transmittance Aggregometry Assay.

Venous blood was obtained from healthy human donors who were drug- and aspirin-free for at least two weeks prior to blood collection or from other species as previously described (S. Mousa et al., *Coronary Artery Disease*, 7 (10): 767-774, 1996). Briefly, blood was collected into citrated Vacutainer tubes. The blood was centrifuged for 10 minutes at 1000 rpm in a Sorvall RT6000 Tabletop Centrifuge with H-1000 B rotor) at room temperature, and platelet-rich plasma (PRP) was removed. The remaining blood was centrifuged for 10 minutes at 2,500 rpm at room temperature, and platelet-poor plasma (PPP) was removed. Samples were assayed on a PAP-4 Platelet Profiler, using PPP as the blank

(100% transmittance). Two hundred microliters of PRP (2-3x10^8 platelets/mL) were added to each micro test tube, and transmittance was set to 0%. Twenty microliters of DMP754 (esterase treated, 100 IU/mL pig liver esterases for 2 hours) or its acid form, XV459, were added at different concentrations for 8 minutes prior to the addition of ADP. Results were expressed as percent inhibition of agonist-induced platelet aggregation or IC_{50} (μM).

D. Human SKBR3 Cell - Vitronectin ($\alpha_v\beta_5$-mediated) Adhesion Assay. The αvβ5-selectivity of this human cancer cell line-mediated adhesion to vitronectin was characterized with an αvβ5 monoclonal antibody. The assay is as previously described (S. Mousa et al., *Coronary Artery Disease*, 7 (10): 767-774, 1996) in which a coaster plate was coated with 100 μL of vitronectin (0.25 μg per well) overnight at 4°C. Following overnight coating, each well was washed twice with 200 μL PBS and non-specific binding was blocked by adding 200 μl of PBS + 50% BSA per well for 1 hour at room temperature. SKBR3 cells or endothelial or smooth muscle cells obtained from various species were detached with 0.005% trypsin/0.1% EDTA, washed, and re-suspended in serum free Mcoy's 5A standard media (Gibco BRL) at 1 x 10^6 cells/mL. Cells were labeled with 2 μM Calcein-AM (Molecular Probes #3100, 50 μg per vial) for 30 minutes at 37°C in a humidified incubator. Following calcein labeling, cells were washed twice with 40 mL of McCoy's 5A media and centrifuged for 15 min at 300 rpm x 2. Cells (1 x 10^6 cells/mL) were preincubated with either 150 μL of test compounds or media, gently mixed, then incubated for 15 minutes at room temperature. Drug treated SKBR3 cells were added to the assay plate in duplicate and incubated for 60 minutes on a shaker at room temperature. The plate was covered with foil to prevent photobleaching of the dye from labeled cells. Following the drug/cell and matrix interaction, media was gently removed from the wells and washed twice with 200 μL McCoy's 5A media. Following washing, 100 μL of McCoy's 5A was added to each well and the flurorescence was read on a Cytofluor 2300 at sensitivity 2, Ex = 485 nM and EM = 530 nm.

Table 3. Assay results for SJ749 inhibitor and analogs.



Incode	HET	R	α5β1 Elisa (nM)	α5β1 Jurkat (nM)	αvβ5 (nM)	αvβ3 B293 (nM)	IIbIIIa PRP (nM)
SJ749	2-pyridyl	Cbz	0.0008	NT	>10,000	49	>100,000
SJ755	2-benzimidazolyl	CO_2nBu	0.39	NT	2200	87	>100,000
SJ754	2-benzimidazolyl	CO_2Me	1.4	NT	110	34	>100,000
SM222	2-imidazolyl	Cbz	13	NT	220	57	17,000
SP742	2-imidazolyl	CO_2nBu	14	NT	>100	130	NT
SP743	2-imidazolyl	CO_2iBu	16	NT	>100	440	NT
SJ756	2-imidazolyl	Cbz	18	NT	>100	97	34,000
SJ756-001	2-imidazolyl	Cbz	NT	720	2100	41	NT
SJ250	2-imidazolyl	CO_2Me	31	NT	>100	57	7,300
SJ757	2-imidazolyl	CO_2CH_2(3-pyr)	39	NT	>100	32	5,600
SJ753	2-benzimidazolyl	benzyl	56	NT	>100	610	NT

Abbreviations: Cbz = carbobenzyloxy; Bu = butyl; Me = methyl; pyr = pyridyl

Example IV: *In Vitro* Studies using Selected Integrin Antagonists

Three peptidomimetic integrin antagonists (DMP 757, XT 199-4, and SJ 749-1) were obtained from Dupont-Merck Pharmaceutical Company, Wilmington, Delaware and tested for their ability to inhibit invasion of A549 cells by *S. pyogenes* strain 90-226. SJ749-1 is (S)-2-[(2,4,6-trimethylphenyl)sulfonyl]amino-3-[[7-benzyloxy carbonyl-8-(2-pyridinylaminomethyl)-1-oxa-2,7-diazaspiro-[4,4]-non-2-en-3-yl]carbonylamino] propionic acid, competes with fibronectin for binding to the α_5B_1 integrin. XT199 is 3-[3-{Imidazolin-2-yl amino)propyloxyl] Isoxazol-5 yl carbonyl amino]-2-phenyl sulfonylamino propionic acid, and is a potent and specific αvB3 integrin atagonist. DMP757 is a meta-aminobenzoic acid cyclic N-methyl RGD analog with high affinity and specificity for the platelet GPIIb/IIIa integrin. It competes with fibrinogen for binding to the GpIIb/IIIa integrin. Varying concentrations of each compound were tested under the following assay conditions, portions of which were described in Example I but which are repeated in this example for clarity:

Bacteria and cell culture. *S. pyogenes* strain 90-226 is a serotype M1 strain cultured from the blood of a septic patient. This strain was obtained from the WHO Center for Reference and Research on Streptococci at the University of Minnesota. *S. pyogenes* strain 90-226 *emm*::Km is an M1⁻ derivative of strain 90-226. *S. pyogenes* strain T25,3 was obtained from Dr. P. M. Schlievert, Department of Microbiology, University of Minnesota.

Bacteria were grown in Todd-Hewitt broth supplemented with 2% neopeptone (THNB, Difco Laboratories, Detroit, MI). Stock cultures were maintained in 15% glycerol, 85% THNB at -80°C. Working cultures were grown by streaking frozen stock cultures onto sheep blood agar plates. Colonies from the blood agar plates were inoculated into THNB and grown for 6 - 8 hours at 37°C. The THNB-grown cultures were diluted 1:100 into fresh THNB and incubated at 37°C for 14 hours. The bacterial cells were harvested by centrifugation at 600 x g for 10 minutes at room temperature. The resulting bacterial pellets were suspended in 1 volume of Hanks balanced salt solution

(HBSS; Life Technologies, Gaithersburg, MD) then recentrifuged. The final pellets were resuspended in HBSS to an OD_{560} of 0.5, then diluted into tissue culture media as described below.

A549 human lung epithelial cells (ATCC CCL 185) were cultured in RPMI-1640 Medium (supplemented with 10% fetal calf serum (Life Technologies)). Cultures of A549 cells were maintained in medium containing 5 μg/ml penicillin and 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO).

Proteins and Reagents. Fetal calf serum was purchased from Life Technologies; Gaithersburg, MD. Purified fibrinogen was obtained Life Technologies, Inc. Fibronectin was dissolved in RPMI to a final concentration of 1 mg/ml, filter sterilized and stored at 5°C. Dimethyl sulfoxide (DMSO) was obtained from Sigma Chemical Co.

The integrin antagonists DMP 757, XT 199-4 and SJ 749-1, were obtained from Dupont-Merk Pharmaceutical Co., Wilmington, DE. Ticlopidine (5-[o-Chlorobenzyl]-4,5,6,7-tetrahydrothieno[3,2-c]pyridine), a GpIIbIIIa antagonist, was purchased from Sigma Chemical Co. 1 mM stock solutions of DMP 757 and XT 199-4 (in 0.1 N HCL) and SJ749-1 (in 50% water, 50% DMSO) were stored at -20°C.. These compounds were dissolved in distilled water at a final concentration of 0.1 M, filter sterilized and stored at -20°C.

Standard epithelial cell invasion assay. Invasion assays were performed as previously described in D. LaPenta et al. (*Proc. Nat'l Acad. Sci., U.S.A.*, 91, 12115-12119), incorporated herein by reference. A549 cells were cultured in 24 well plates in RPMI, 10% FCS without antibiotics. Confluent monolayers were infected with 1-5 x 10^5 bacterial cfu suspended in 0.5 ml of RPMI containing 25 μg/ml of fibrinogen/fibronectin (RPMI-Fg/Fn). The candidate antagonists were diluted directly into RPMI-Fg/Fn prior to addition of bacterial cells. The final concentrations of antagonists were varied from 60 nM to 200 μM. In control experiments (i.e. no antagonist addition) invasions were performed in RPMI-Fg/Fn containing a level of antagonist solvent (HCl for experiments performed with DMP 757 and XT 199-4, DMSO in SJ749-1 experiments and distilled water in experiments performed with ticlopidine) equivalent to that present in medium containing an antagonist.

Plates containing infected monolayers were centrifuged at 200 x g for 5 minutes at room temperature, then incubated for 2 hours at 37°C in 5% CO_2/95% air. Monolayers were then washed 3 times with 1 ml of HBSS and 1 ml of RPMI containing 10% FCS, 100 μg/ml gentamicin and 5 μg/ml penicillin, was added to each monolayer. Following 2 hours incubation at 37°C, the monolayers were washed 3 times with HBSS, dispersed by the addition of 0.2 ml of 0.25% trypsin, 1 mM EDTA (Life Technologies), then lysed by dilution into 0.8 ml of sterile, distilled H_2O. The numbers of bacterial cfu released from the lysed epithelial cells were determined by dilution of lysates in HBSS then plating on Todd-Hewitt agar (Difco).

In some experiments, the total numbers of cfu in wells was determined after the first two hour incubation. Culture media were removed from the monolayers and transferred to 1.5 ml microcentrifuge tubes. 0.2 ml of trypsin-EDTA was then added to the wells and incubated with the monolayers for 10 minutes at room temperature. 0.8 ml of sterile distilled H_20 was added to the trypsinized cells, the solutions were removed from the wells and transferred to the tubes containing the culture medium. The suspensions were then diluted in HBSS and plated. The total numbers of cfu were used to determine the extent of bacterial growth during the invasion period and to calculate the percentages of adherent cfu.

To measure bacterial adherence, culture media were removed from monolayers at the end of the invasion period and discarded. The monolayers were then washed 3 times with HBSS to remove nonadherent bacteria. Epithelial cells were dispersed and lysed and bacteria were plated as described above. While the numbers of cfu recovered from these wells is reflective of the number of adherent and internalized cfu, for simplicity, we will refer to these bacteria as adherent cfu.

To determine the effects of SJ 749-1 on adherence of A549 cells to tissue culture wells, 0.5 ml of RPMI-Fg/Fn and RPMI-Fg/Fn containing SJ 749-1 were added to monolayers. The plates were centrifuged then incubated at 37°C for 2 hours. Media were then removed from the wells, the monolayers were washed 3 times with 1 ml of HBSS and 1 ml of RPMI containing antibiotics was added to each well. Following a second 2 hour incubation, the monolayers were washed 3

times with HBSS and dispersed by the addition of 0.2 ml of 0.25% trypsin, 1 mM EDTA (Life Technologies). The dispersed cells were suspended in 0.8 ml of HBSS and portions were diluted 1:1 into 0.4% Trypan Blue (Sigma Chemical Co.) in HBSS and the mixtures were incubated for 5 minutes at room temperature. Cells were enumerated by transferring portions of the Trypan Blue suspensions to a haemocytometer chamber and viewing by light microscopy at a magnification of 100X.

Results. Neither DMP 757 (an αIIbβ3 antagonist) nor XT199-4 (an αvβ3 antagonist) were found to affect invasion even at concentrations as high as 200 μM (Fig. 8). Compound SJ 749-1 was found to inhibit invasion when present at concentrations >1.5 μM (Fig. 9). Intracellular invasion was completely inhibited by >40 μM of SJ 749-1. SJ 749-1 has no apparent toxic effect upon the cultured epithelial cells. Control experiments, however, did indicate that the solvent used for suspension of the drug (50% DMSO, 50% water) can cause dissociation of the epithelial cells from the substrate, when present at high concentrations. As a result, subsequent experiments were performed in the presence of 5 μM of the integrin antagonist. When this concentration of drug is used, the level of solvent present has no apparent effect on the cultured cells (Fig. 10), but the antagonist still inhibits intracellular invasion by approximately 80%. No direct effect of SJ 749-1 on bacterial viability was observed (Fig. 10). In Fig. 10, relative number of cells is defined as the ratio of the number of bacterial cfu or A549 cells present in wells containing a given concentration of SJ749-1, divided by the number of bacterial cfu or A549 cells present in wells to which no drug was added.

Experiments were performed to determine which step(s) of the invasion process are affected by SJ 749-1 (Table 4). As noted above, 5 μM SJ 749-1 reduces the efficiency of intracellular invasion by approximately 5-fold and has no obvious effect on bacterial growth. Bacterial adherence to A549 cells is also unaffected by the antagonist. Thus, SJ 749-1 directly affects the efficiency by which bacteria are internalized by host cells.

Table 4. Invasion steps affected by the integrin antagonist SJ 749-1

Assay Condition[a]	% Invasion[b]	% Adherence[c]	Growth Index[d]	Invasion Index[e]
0.25% DMSO	11.9 (8.6-16.0)	10.9 (9.1-12.5)	13.3 (11.5-15.0)	0.08 (0.05-0.10)
0.25% DMSO, 5 mM SJ 749-1	2.7 (2.4-3.0)	13.9 (13.1-14.8)	11.0 (8.8-13.2)	0.01 (0.008-0.02)

[a] Invasions were performed in RPMI-Fg containing 5 uM SJ749-1, 0.25% DMSO and in RPMI-Fg/Fn containing 0.25% solvent (DMSO). All values are the means and ranges (in parentheses) from assays performed in triplicate.

[b](Internalized cfu/cfu in the inoculum) x 100

[c](Adherent plus internalized cfu/total cfu present at the end of the invasion incubation) x 100.

[d]Total cfu present at the end of the invasion incubation/cfu in the inoculum.

[e]Internalized cfu/adherent plus internalized cfu.

Consistent with this result are the findings that the ability of SJ749-1 to inhibit invasion is dependent upon the presence of fibronectin and bacterial expression of M1 protein (Table 5). We propose that SJ749-1 inhibits invasion by interfering with the interactions between M1 protein, fibronectin and epithelial cells. This could be accomplished by either 1) interfering with fibronectin binding to M1 protein, 2) blocking an interaction between fibronectin-bound M1 protein and an epithelial cell receptor or 3) blocking an interaction between bacteria-bound fibronectin and a host receptor.

Table 5. Inhibition of intracellular invasion by SJ 749-1: dependence upon fibrinogen/fibronectin and M1 protein

S. pyogenes strain	Assay Medium	Drug[a]	% Invasion[b]
90-226	RPMI	none	0.64 (0.52-0.74)
"	"	SJ 749-1	0.79 (0.72-0.85)
"	RPMI-Fg/Fn	none	11.6 (8.8-16.1)
"	"	SJ 749-1	2.3 (2.1-2.4)
90-226 *emm*::Km	RPMI-Fg/Fn	none	0.14 (0.08-0.22)
"	"	SJ 749-1	0.48 (0.43-0.52)

[a]SJ749-1 was added to media to a final concentration of 5 mM. DMSO was present in all media at a final concentration of 0.25%.

[b](Internalized cfu/cfu in the inoculum) x 100. All values are the means and ranges (in parentheses) from assays performed in triplicate.

The GpIIbIIIa antagonist ticlopidine (Di Minno et al., *J. Clin. Invest.*, 75, 328-338 (1985) were also found to inhibit intracellular invasion by strain 90-226 (Fig. 11). Ticlopidine reduced invasion by approximately 3-fold at concentrations >50 μM. Additional candidate compounds can be tested for efficacy in the inhibition of intracellular invasion by *S. pyogenes* strain 90-226 using the assay as described in this example. The assay can likewise be used to assess the efficacy of selected candidate compounds on intracellular invasion by other clinical or laboratory isolates of *S. pyogenes*, and can be readily modified by one of skill in the art to test for inhibition of intracellular invasion by other known ECM protein-binding pathogenic microbes, using the appropriate ECM protein as the competitive ligand.

Example V. Interaction of *S. pyogenes* with Primary Cultures of Tonsillar Epithelial Cells

Primary cultures of tonsillar epithelial (PTE) cells were cultured from surgically-removed tonsils by Dr. P. Southern, Department of Microbiology, University of Minnesota. Tonsil-derived cells grew as monolayers *in vitro* and exhibited, predominately, an epithelial cell-like morphology. PTE cells were found to express E-cadherin and cytokeratins, thus confirming their epithelial origin. Cells were also found to express integrins β1 and α5β1. No indigenous colony-forming bacteria were detected in the cultures after four weeks growth in antibiotic containing medium.

For performance of invasion experiments, wells containing 1-2 x10^5 PTE cells were infected with 2x10^5 CFU of *S. pyogenes*. Specifically, *S. pyogenes* strains 90-226 ($M1^+$, open bars) and 90-226 *emm*1::Km ($M1^-$, hatched bars) were suspended in RPMI medium containing 10% FBS (RPMI-FBS), then inoculated onto monolayers of second passage cultures of tonsillar epithelial cells (PTE) cells. Assays of bacterial adherence and internalization were performed as described in Example I for A549 cells, and the results are shown in Fig. 12. All assays were performed in triplicate with two different passages of PTE cells. Percent bacterial survival is expressed as the percentages of CFU in the

inoculum that survived antibiotic selection. Percent adherence is expressed as the percentages of total CFU that remained associated with monolayers after three successive washes with buffer. Data are the mean percentages of assays performed in triplicate.

Invasion of PTE cells was found to be dependent upon bacterial expression of M1 protein. Moreover, as previously found for A549, HEp-2 and HeLa cells, M1 expression stimulated adherence by approximately 2.5-fold, when assays were performed in the presence of FBS (Fig. 12). Internalization of $M1^+$ streptococci was further shown to be heavily dependent upon the presence of invasion agonists (Fig. 13). Specifically, *S. pyogenes* 90-226 was suspended in RPMI medium containing no agonists (designated "None" in Fig. 13) or in medium containing 10% FBS, 10 μg/ml human serum fibronectin, or 10 μg/ml mouse laminin, as indicated, just prior to infection of monolayers. Data are the mean percentages of assays performed in triplicate with third (open bars) or fifth (hatched bars) passage PTE cells. Percent bacterial survival was calculated as described for Fig. 12. Addition of FBS, fibronectin or laminin stimulated uptake of $M1^+$ bacteria by about 118-, 244-, and 93-fold, respectively.

Previous experiments in our laboratory showed that the RGD mimic compound, SJ749-1 (Example IV), and anti-integrin monoclonal antibodies (Example I) can inhibit the uptake of streptococci by A549 cells. Experiments were performed to determine whether these same integrin antagonists could inhibit invasion of PTE cells by streptococci, thereby increasing their sensitivity to antibiotics (Fig. 14). Specifically, *S. pyogenes* 90-226 was suspended in RPMI-FBS containing the indicated integrin antagonists, then inoculated onto monolayers of fourth (open bars) or fifth (hatched) passage PTE cells. Integrin antagonists were present at the following concentrations: SJ749-1, 50 μM; anti-integrin α5β1 mAb, 0.35 μg/ml; and anti-integrin β1 mAb, 0.5 μg/ml. Anti-integrin β2 mAb (1.8 μg/ml) was included in the experiment as a negative control. Data are the means of experiments performed in triplicate. Percent bacterial survival was calculated as described for Fig. 12. As seen in Fig. 14, SJ749-1 and mAbs reacting with integrins β1 or α5β1 inhibited internalization of streptococci and increased their sensitivity to a combination of penicillin and

gentamicin. SJ749-1 had no effect on bacterial survival in the absence of antibiotics.

These experiments validate the relevance of previous studies performed with immortalized human epithelial cell lines. Internalization of *S. pyogenes* by primary tonsillar epithelial cells is M1 dependent and makes use of the same extracellular matrix proteins and integrins as established cell lines, such as A549 monolayers.

Example VI. Inhibition of Intracellular Invasion by *S. pyogenes* by Inhibitors of Host Cell Signal Transduction

Numerous microbial pathogens, including *S. pyogenes*, exploit host integrins for entry into non-phagocytic cells. Integrin-mediated uptake of microorganisms typically occurs via multi-step pathways involving: 1) microbial engagement of receptors; 2) host cell signal transduction via integrin clustering or conformational changes and phosphorylation of intracellular macromolecules; and 3) reorganization of the host cell cytoskeleton. Activation of such pathways results in the eventual endocytosis of adherent microbes.

Compounds that interfere with any one of these steps could potentially block intracellular invasion by streptococci, thereby increasing the extent of bacterial killing by antibiotics. Compounds that inhibit ligand binding by integrins (i.e. SJ749-1 and anti-integrin mAbs) or actin polymerization, e.g., cytochalasin D (D. LaPenta et al., *Proc. Natl. Acad. Sci. USA*, **91**, 12115-12119 (1994)) can inhibit invasion by *S. pyogenes*. Inhibition of signal transduction can block the cytoskeletal and other physiological changes required to initiate endocytosis. Therefore, inhibitors of integrin-initiated signal transduction were tested to further substantiate the idea that inhibition of internalization can increase the capacity of antibiotics to eliminate streptococci from potential epithelial reservoirs. Kinase inhibitors were serially diluted in RPMI-FBS then added to A549 cells. Drugs were incubated with monolayers for 30' at 37 C prior to the addition of streptococci and the presence of the drugs was maintained during the invasion period. Percent bacterial survival was determined as described above and compared to that for untreated A549 cells (100% survival).

All values are the averages from duplicate wells. None of the inhibitors was found to adversely affect bacterial viability or adherence of epithelial cells to culture wells.

Staurosporine, an inhibitor of several phosphotyrosine, phosphoserine and phosphothreonine kinases (I. Rosenshine et al., *Methods Enzymol.*, **236**, 467-476 (1994)) inhibits invasion of A549 cells in a dose-dependent manner (Fig. 15). Addition of 1 μM of the inhibitor reduced bacterial internalization by >90%. Genistein, a specific inhibitor of tyrosine kinases (I. Rosenshine et al., *Methods Enzymol.*, **236**, 467-476 (1994)), inhibited invasion when present at high concentrations. The phosphatidylinositol-3 kinase inhibitor, wortmannin (I. Nakamura et al., *FEBS Lett.*, **361**, 79-84 (1995)), is a potent invasion inhibitor; 22 nM of the drug reduced invasion by >90%. These results implicate the activities of host cell kinases in the pathway leading to internalization of streptococci. None of the compounds are toxic for the bacteria in the absence of antibiotics. Although these particular compounds are too toxic for use as chemotherapeutic agents, our results confirm that inhibitors of bacterial internalization do increase the sensitivity of streptococci to antibiotics.

Example VII. Anti-integrin compound SJ749-1 can increase the sensitivity of *S. pyogenes* to killing by penicillin

This experiment tested the impact of the anti-integrin SJ749-1 on the sensitivity of streptococci to increasing doses of penicillin. *S. pyogenes* 90-226 was suspended in RPMI-FBS (Fig. 16, A549 cells) and in the same medium containing, additionally, 5 μM SJ749-1 (Fig. 16, A549 cells, SJ749-1), then inoculated onto monolayers of A549 cells. Following incubation for 2 hours at 37 C, infected monolayers were washed and media containing 5 to 100 μg/ml of penicillin was added to the wells. Thereafter, the standard invasion assay procedure was followed. Values plotted in the Fig. 16 are the mean percentages of assays performed in duplicate.

To verify killing of extracellular bacteria, streptococci were exposed to antibiotic in the absence of A549 cells (No A549 cells). For this portion of the experiment, bacteria were inoculated into RPMI-FBS and incubated for two

hours at 37 C. The culture was then aliquoted and varying concentrations of penicillin (0 – 20 μg/ml) were added to individual tubes. Following a second 2 hour incubation, bacteria were harvested by centrifugation, washed, resuspended in ½ volume buffer and plated onto THA.

In the absence of epithelial cells, ≥1 μg/ml of penicillin was sufficient to eliminate >99.8% of streptococci. In the presence of epithelial cells, 4-8% of the inoculated streptococci survived 2 hours incubation in as much as 100 μg/ml of penicillin. The highest concentration tested is 1000 times greater than the minimal inhibitory concentration of penicillin for *S. pyogenes* and approximately 500 times the peak concentration achieved in a person's serum following oral administration of the antibiotic. Anti-integrin SJ749-1 increased the sensitivity of streptococci to penicillin from 1.5 to 3.7 fold, depending on the penicillin concentration. It is anticipated that SJ749-1 would further increase the sensitivity of streptococci to penicillin, if exposure to this combination of anti-integrin and penicillin were prolonged.

Example VIII. Inhibitory Compounds as Broad-Spectrum Enhancers of Antibiotics

A. Strep throat.

Depending on the time of year Strep throat accounts for 10-30% of all office visits in a general medical practice. Penicillin is the antibiotic of choice for treatment of Strep throat and more than four million prescriptions of this antibiotic are prescribed for this infection in the U.S. annually. Erythromycin and clindamycin are the second and third choice, and prescribed less often (M. Pichichero, *Ann. Emerg. Med.*, **25**, 404-406 (1995); I. Brook et al., *Laryngoscope*, **96**, 1385-1388 (1986)). Persistent and recurrent throat infections following antibiotic therapy are common in children and occasionally a problem for adults, particularly those in military camps (C. Timon et al., *Respir. Med.*, **84**, 395-400 (1990); M. Gerber et al., *Pediatr. Inf. Dis. J.*, **13**, 576-579 (1994); G. Gray et al., *N. Engl. J. Med.*, **325**, 127-8 (1991)). Genetic resistance to erythromycin and tetracycline have been reported, but this species has remained exquisitely sensitive to penicillin by in vitro assays. The means by which

streptococcus resist penicillin and continue to be carried in the throat is unknown (E. Kaplan et al., *J. Lab. & Clin. Med.*, **98**, 326-35 (1981)). We postulate, however, that streptococci reside inside tonsilar cells where they are concealed from penicillin. Intracellular streptococci are resistant to more than 100μg/ml penicillin (Table 6); whereas, the therapeutic concentrations in tissue are less than 0.5 μg/ml. Moreover, *S. pyogenes* can be readily cultured from tonsils that have been surgically removed following vigorous antibiotic treatment (I. Brook et al., *Laryngoscope*, **96**, 1385-1388 (1986); A. Stjernquist-Desatnik et al., *J. Laryng. and Otol.*, **105**, 439-41 (1991)). ENT physicians have long recognized that the removal of tonsils usually ends the cycle of recurrent Strep throat suffered by some individuals. Life threatening streptococcal wound infections, necrotizing fasciitis and other soft tissue infections have also been reported to resist penicillin therapy.

Table 6

Intracellular Streptococci are Highly Resistant to Penicillin

Penicillin Concentration	Number of Streptococci associated with epithelial cells	Percent Streptococci which survive 2 hrs. incubation with penicillin*
0	$7.5x10^3$	100
5	$4.6x10^4$	6.0
10	$3.8x10^4$	5.1
50	$4.7x10^4$	6.2
100	$8.4x10^4$	11.2

*No Streptococci survive without epithelial cells

Osterlund et al. (Laryngoscope, 107, 640-646 (1997) have explained the persistance of streptococci after penicillin therapy. They showed that 14 out or 15 tonsils excised from children with recurrent tonsilitis retain streptococci within epithelial and macrophage-like cells. Tonsils removed for problematic snoring did not have streptococci associated with them.

In vitro data predicts that compounds that can interrupt the intracellular-extracellular cycle of streptococcal infections will improve the efficacy of penicillin therapy for Strep throat and *S. pyogenes* infection of soft tissue. Therefore, compounds such as SJ749-1 or related compounds are advantageously included in formulations of penicillins, cephalosporins, and aminoglycosides--antibiotics that are unable to effectively penetrate mammalian cells. The optimal concentration of inhibitory compound in drug combinations with antibiotics can be determined by routine investigations of their solubility and achievable tissue concentration. The inhibitory compound/antibiotic formulations can be administered orally, by intramuscular injection (e.g. Benzathine penicillin), or throat lozenge, mouthwash or gargle for local coverage. The standard course of oral penicillin treatment of ten days, which is well-known in the art, may be shortened by inclusion of inhibitory compounds in the formulation.

B. Prophylaxis of bacterial endocarditis. Infective endocarditis is primarily caused by various streptococcal species, enterococci and coagulase negative staphylococci. These bacteria bind to fibronectin and fibrin vegetations on heart valves and the endocardium and are transiently released into the blood stream. Penicillin frequently failed to protect patients who had valve abnormalities and underwent various invasive medical procedures (D. Durack et al., *J. Am. Med. Assoc.*, **250**, 2318-22 (1983)). Moreover, penicillin and aminoglycoside - penicillin combinations treatment regiments for endocarditis require long, vigorous periods of therapy to be eliminated, and still frequently reoccur . Many of these species have been shown to invade both endothelial and epithelial cells *in vitro*. Since neither penicillins or aminoglycoside are able to penetrate these cells, it is reasonable to postulate that the intracellular states of these bacteria may account for the resistant nature of these infections to

antibiotic therapy. Combinations of inhibitory compounds, penicillin and gentamicin or other aminoglycosides could more rapidly and completely eliminate these recalcitrant infections.

Hospital-based bacterial infection. Nasal and/or rectal carriers of highly virulent strains of *Staphylococcal aureus* and *Streptococcus pyogenes* can be responsible for serious nosocomial infections. Hospital personnel associated with this problem can be required to miss work until they are free of incriminating organisms. Elimination of the carrier state requires prolonged antibiotic therapy and can be difficult to accomplish. Although the biology of this problem is not well understood it is reasonable to postulate that these bacteria cycle between intracellular-antibiotic resistant and extracellular-antibiotic sensitive states. Again, compounds that prevent microbial internalization may interrupt this cycle and improve the efficacy of antibiotics for eliminating the carrier state.

Otitis media. 70 % of the amoxicillin prescribed in the U.S. is for otitis media in children between the ages of 1-5 years. Recurrent middle ear infections are the most common and persistent infection presented to pediatricians in developed countries. *Streptococcus pneumoniae* are one of the most common causes of recurrent middle ear disease. Although genetic resistance is on the rise, most strains are sensitive to amoxicillin and other penicillins *in vitro*. The biological basis for recurrent infection and the physical location of these bacteria between episodes of disease are not known. *S. pneumoniae* is, however, able to bind fibronectin, and could, therefore, be internalized by epithelial cells lining the middle ear. RGD peptidomimetics or other compounds that compete for the fibronectin binding site on the bacteria or epithelial mucosa could be designed which will inhibit uptake of these bacteria. Combinations of these compound with the appropriate antibiotic could improve therapy or eliminate recurrent middle ear infections in children.

The complete disclosure of all patents, patent documents, and publications cited herein are incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not

limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claim.

WHAT IS CLAIMED IS:

1. A method for treating a mammal infected by a pathogenic microbe comprising administering to the mammal a therapeutic composition comprising an inhibitory compound in an amount effective to inhibit adherence to or invasion of a cell of the mammal by the pathogenic microbe, wherein the inhibitory compound is a fibronectin antagonist that binds to an $\alpha 5\beta 1$ integrin.

2. The method of claim 1 wherein the fibronectin antagonist is selected from the group consisting of a cyclic RGD-containing peptide, a non-peptide, and an antibody.

3. The method of claim 2 wherein the inhibitorycompound is a synthetic peptidomimetic non-peptide.

4. The method of claim 1 wherein the therapeutic composition further comprises a pharmaceutically acceptable carrier.

5. The method of claim 1 wherein the pathogenic microbe is bacterial pathogen.

6. The method of claim 5 wherein the bacterial pathogen is a member of a genus selected from the group consisting of *Streptococcus*, *Enterococcus*, *Yersinia*, *Salmonella*, *Chlamydia*, *Listeria*, *Shigella*, *Neisseria*, *Mycobacterium*, *Staphylococcus*, *Prevotella*, *Porphyromonas*, *Helicobacter* and *Legionella*.

7. The method of claim 6 wherein the bacterial pathogen is a streptococcus.

8. The method of claim 1 wherein the pathogenic microbe is a fungus.

9. The method of claim 8 wherein the fungus is selected from the group consisting of *Candida*, *Histoplasma* and *Aspergillus*.

10. The method of claim 1 wherein the pathogenic microbe comprises an invasin.

11. The method of claim 1 wherein the mammalian cell is a nonphagocytic cell.

12. The method of claim 1 further comprising administering at least one antibiotic in an amount effective to treat the pathogenic microbe.

13. The method of claim 12 wherein the at least one antibiotic is penicillin.

14. The method of claim 12 further comprising administering an effective amount of a protein synthesis inhibitor.

15. The method of claim 12 further comprising administering an effective amount of an inhibitor of a microbial or host cell enzyme capable of degrading or otherwise inhibiting the activity of the at least one antibiotic.

16. A composition for treatment of a mammal infected by a pathogenic microbe comprising:

(a) an inhibitory compound in an amount effective to inhibit adherence to or invasion of a cell of the mammal by the pathogenic microbe, wherein the inhibitory compound is a fibronectin antagonist that binds to an $\alpha 5\beta 1$ integrin;

(b) at least one antibiotic effective to treat the pathogenic microbe; and

(c) a pharmaceutically acceptable carrier.

17. The composition of claim 16 wherein the fibronectin antagonist is selected from the group consisting of a cyclic RGD-containing peptide, a non-peptide, and an antibody.

18. The composition of claim 16 wherein the at least one antibiotic is penicillin.

19. The composition of claim 16 further comprising an effective amount of a protein synthesis inhibitor.

20. The composition of claim 16 further comprising an effective amount of an inhibitor of a microbial or host cell enzyme capable of degrading or otherwise inhibiting the activity of the antibiotic.

21. The composition of claim 20 wherein the at least one antibiotic is penicillin and the inhibitor of a microbial or host cell enzyme capable of degrading or otherwise inhibiting the activity of the antibiotic is a penicillinase inhibitor.

22. A method for identifying inhibitory compounds effective to inhibit adherence to or invasion of a mammalian cell by a pathogenic microbe comprising:

(a) exposing a mammalian cell culture to a pathogenic microbe in the presence of fibronectin and a candidate inhibitory compound effective to inhibit binding of fibronectin to an $\alpha 5\beta 1$ integrin, for a time effective to allow adherence to or invasion of the mammalian cell by the pathogenic microbe; and

(b) assaying the cell culture to determine the presence of internalized or adhered pathogenic microbe.

Fig. 1

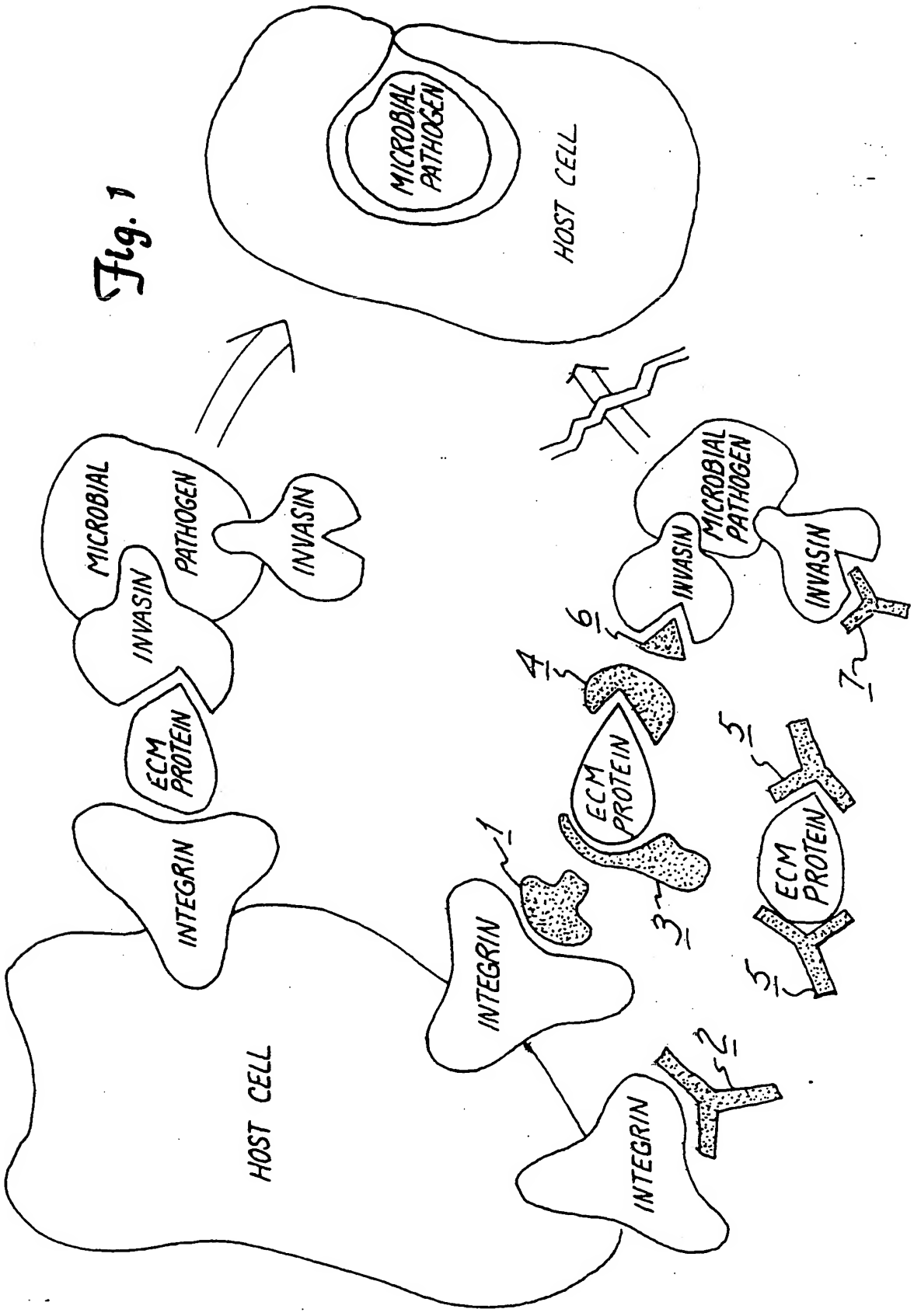

MICROBIAL PATHOGEN
HOST CELL
MICROBIAL PATHOGEN
INVASIN
ECM PROTEIN
INTEGRIN
HOST CELL
INTEGRIN
ECM PROTEIN
INVASIN
MICROBIAL PATHOGEN
INVASIN
1
2
3
4
5
6
7

Fig. 2a

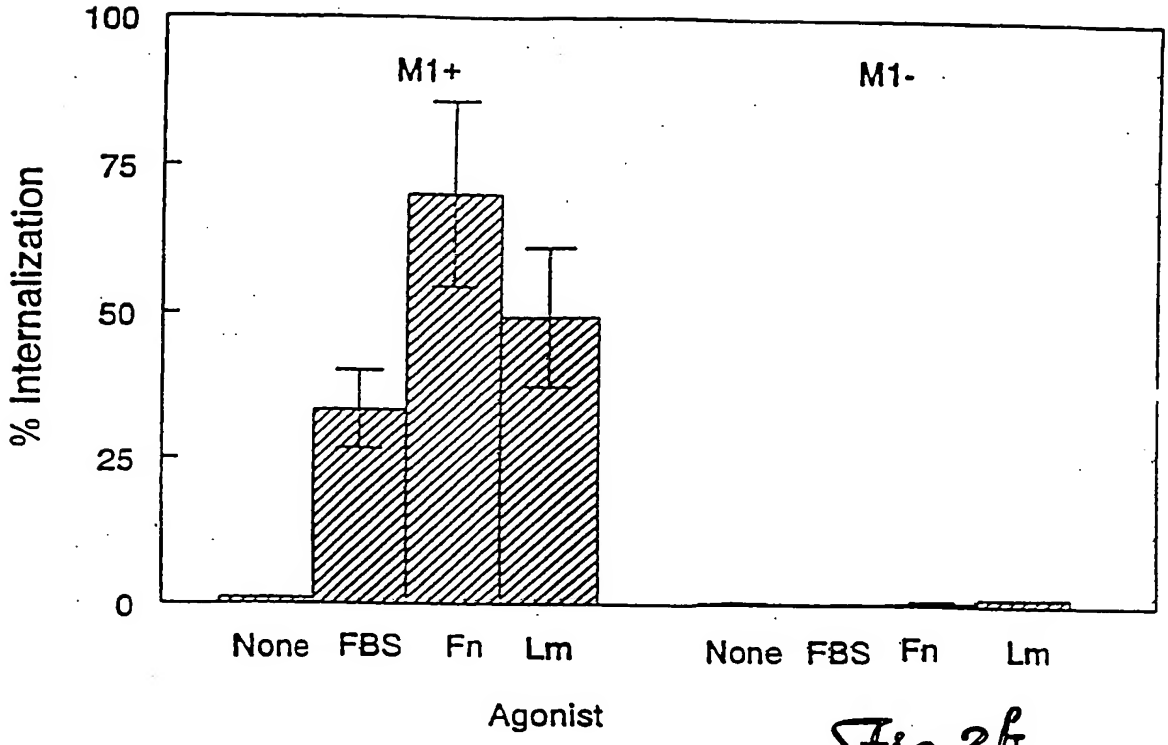
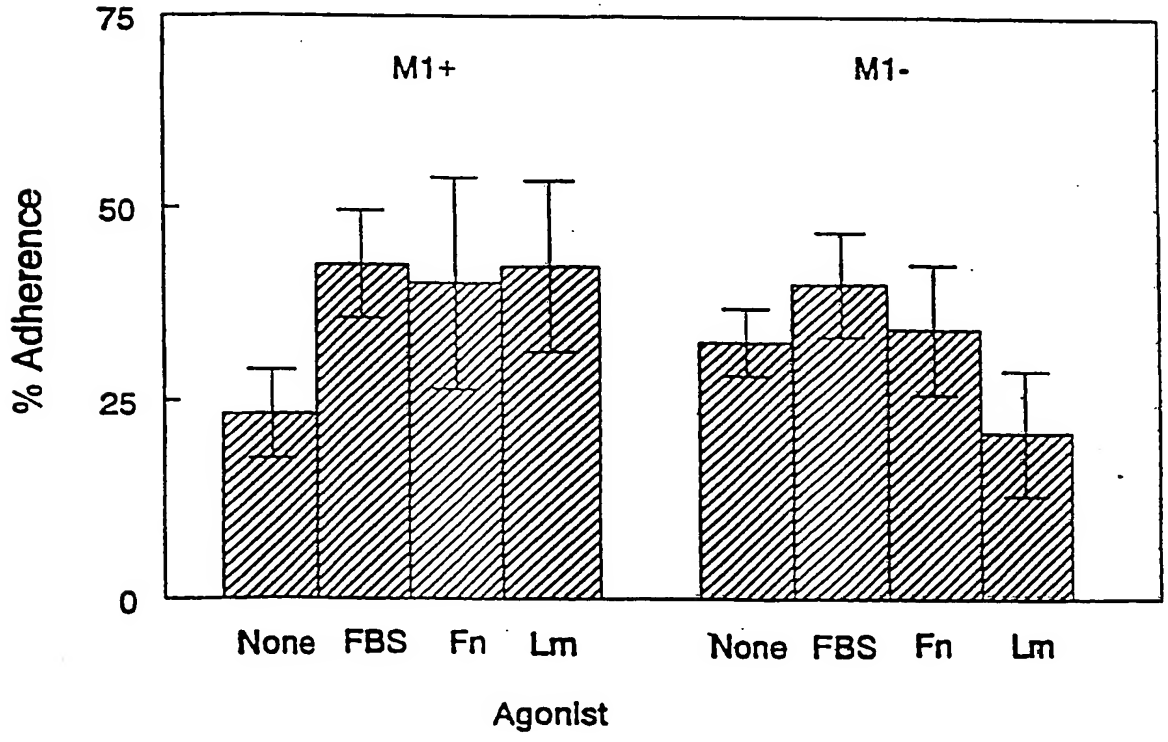

100
75
50
25
0
% Internalization
M1+
M1-
None FBS Fn Lm
None FBS Fn Lm
Agonist

Fig. 2b


75
50
25
0
% Adherence
M1+
M1-
None FBS Fn Lm
None FBS Fn Lm
Agonist

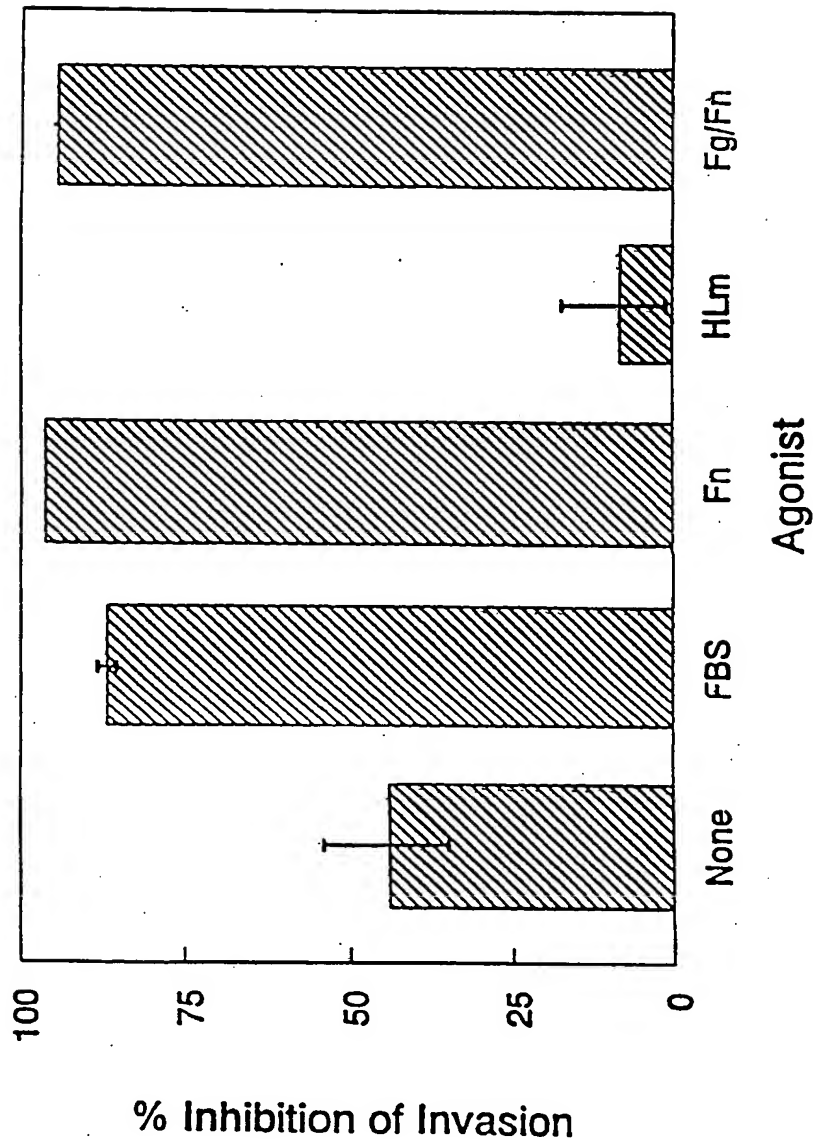

100
75
50
25
0
% Inhibition of Invasion
None
FBS
Fn
HLm
Fg/Fh
Agonist

Fig. 3

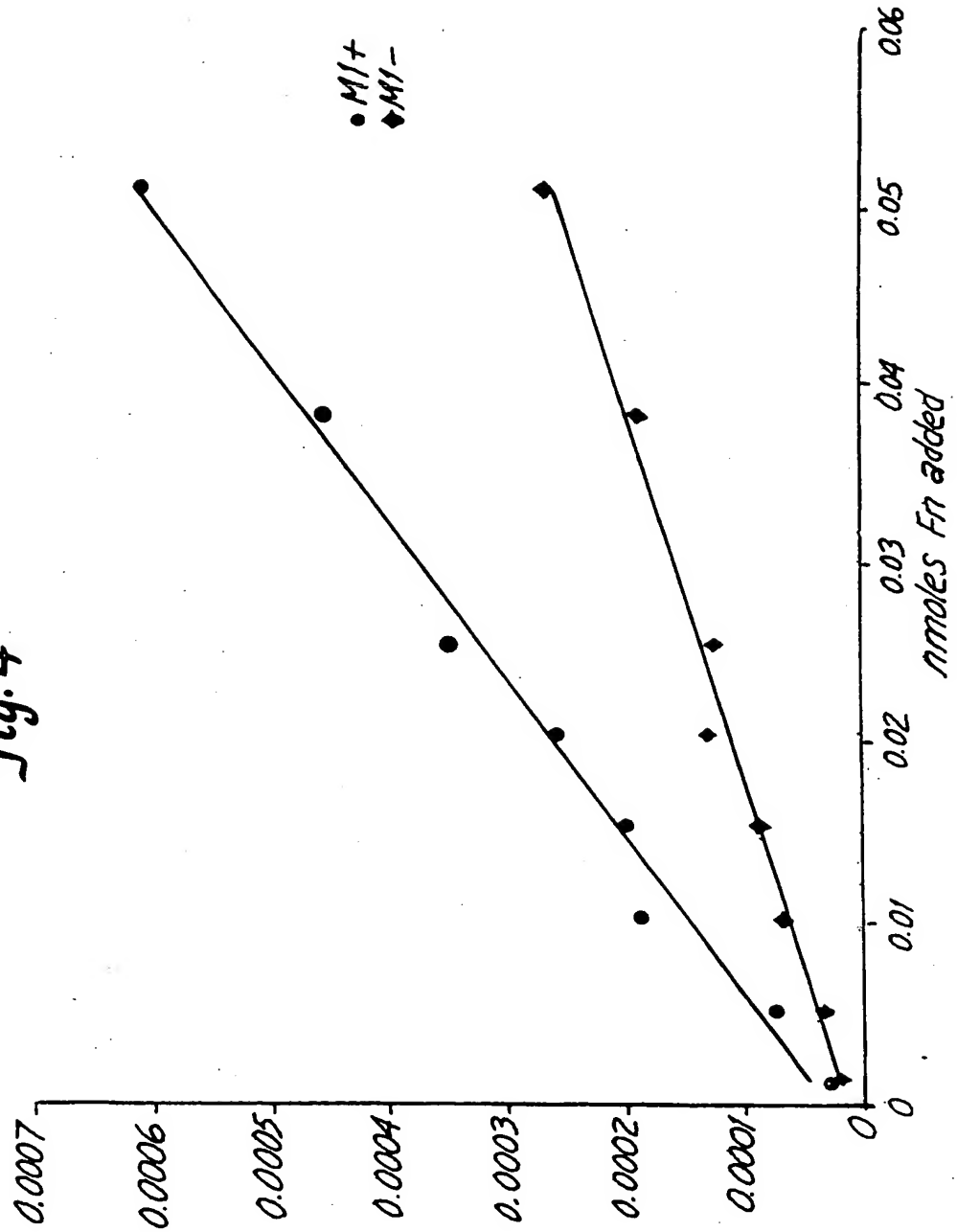

M1+
M1-
0.0007
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0
0.01
0.02
0.03
0.04
0.05
0.06
nmoles Fn added

Fig. 4

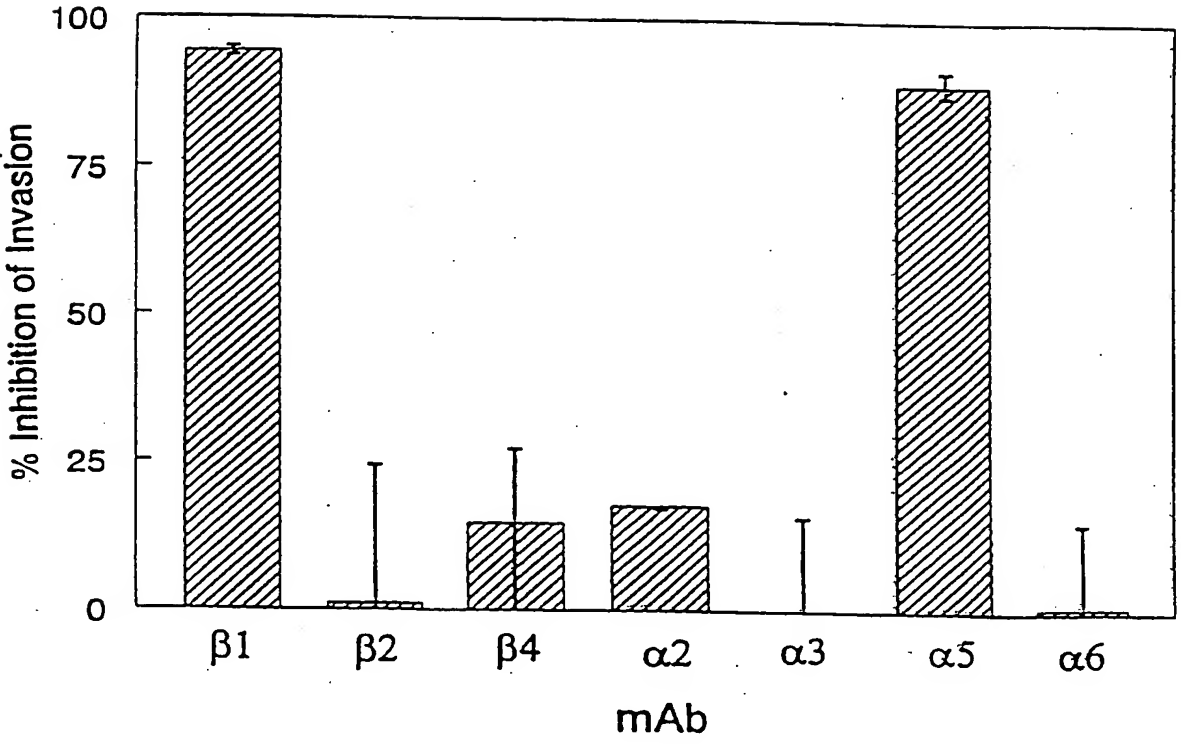


Fig. 5

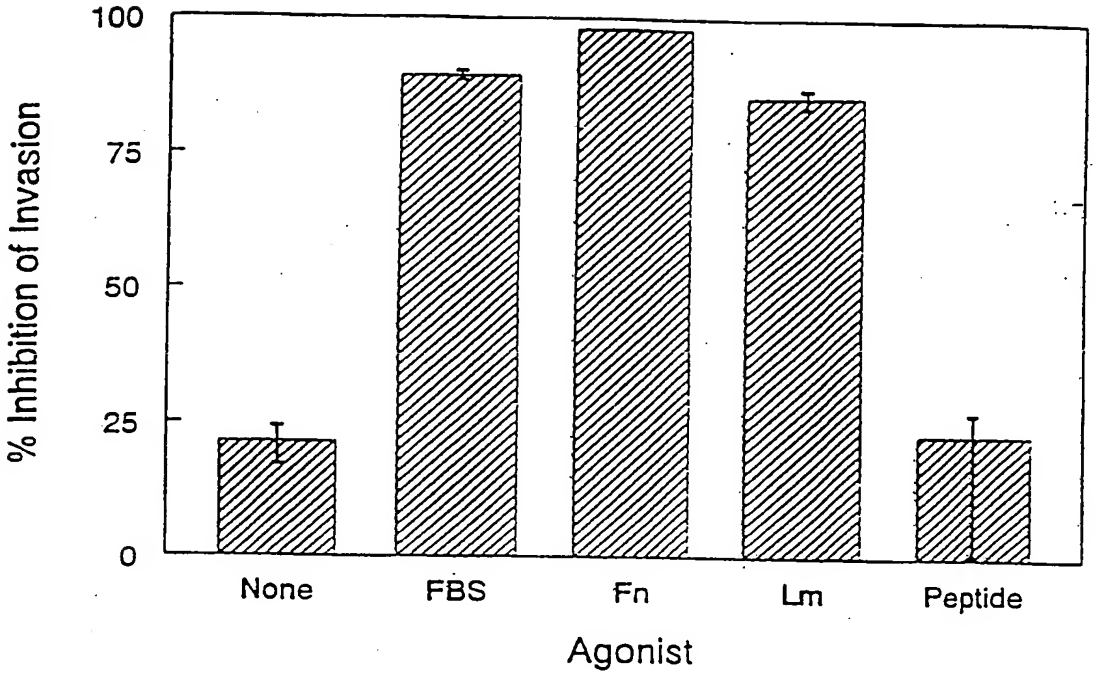

100
75
50
25
0
% Inhibition of Invasion
None
FBS
Fn
Lm
Peptide
Agonist

Fig.6a

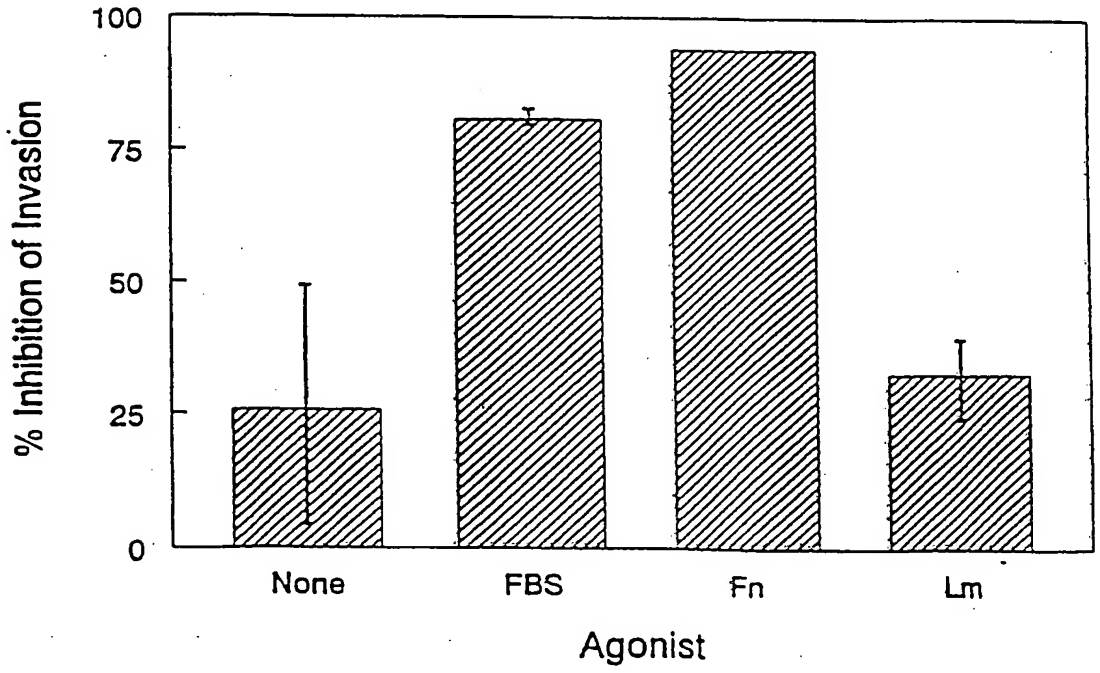

100
75
50
25
0
% Inhibition of Invasion
None
FBS
Fn
Lm
Agonist

Fig.6b

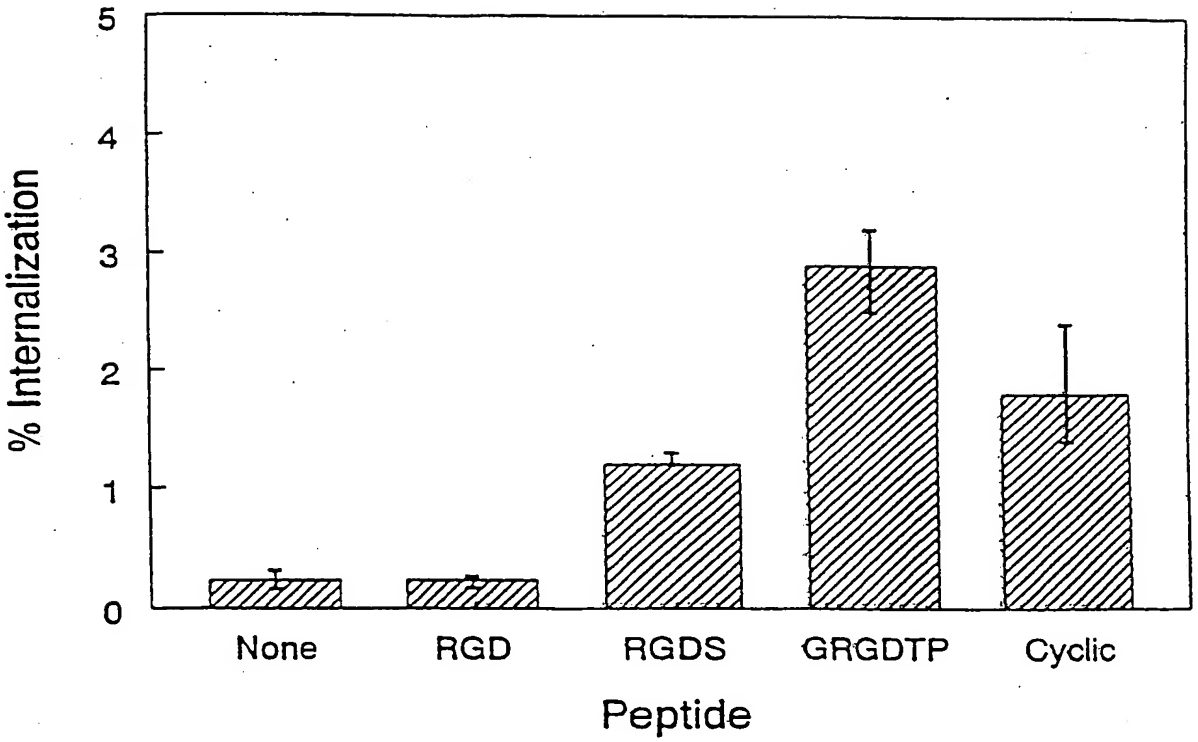

5
4
3
2
1
0
% Internalization
None
RGD
RGDS
GRGDTP
Cyclic
Peptide

Fig. 7

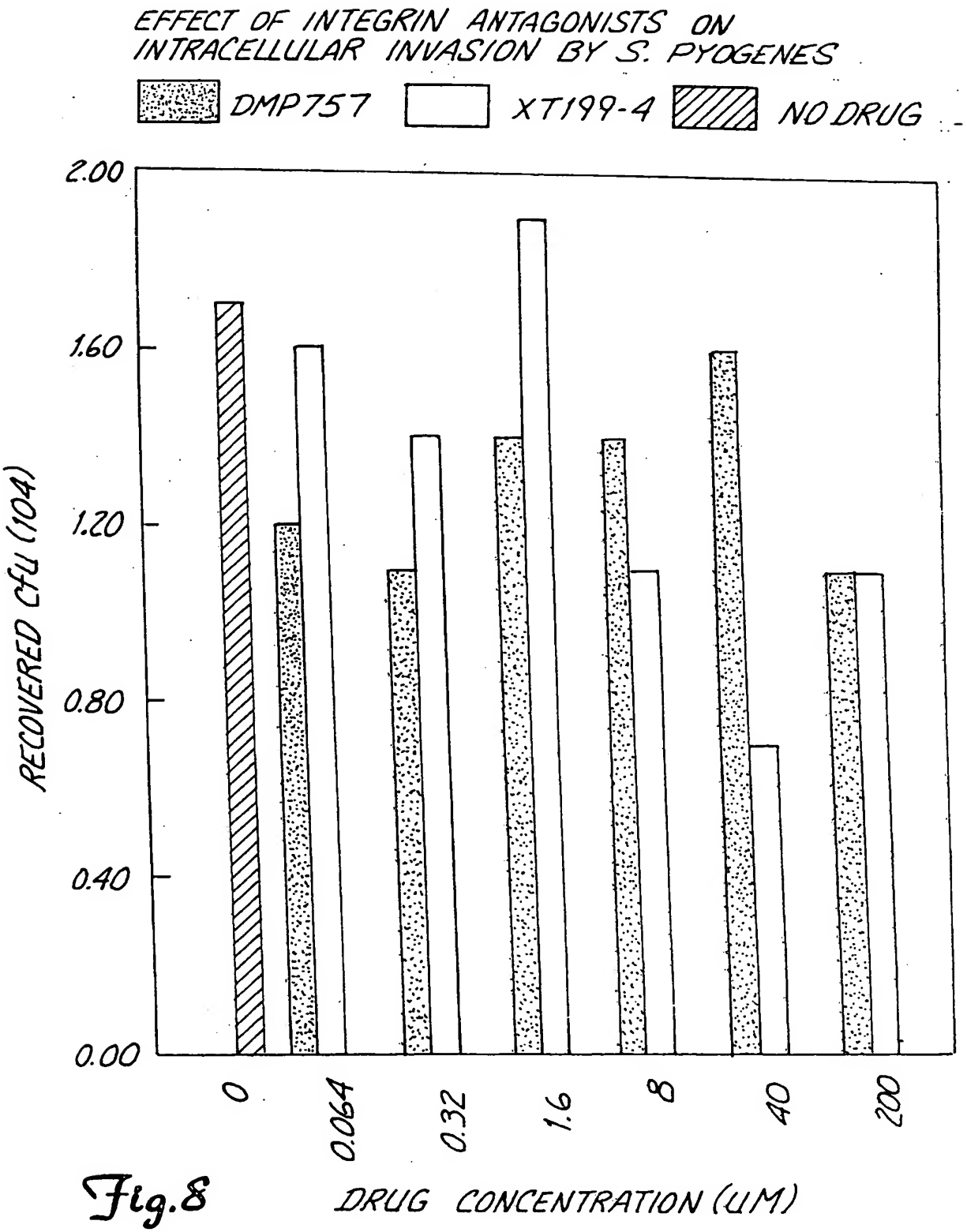

EFFECT OF INTEGRIN ANTAGONISTS ON
INTRACELLULAR INVASION BY S. PYOGENES
DMP757
XT199-4
NO DRUG
RECOVERED CFU (104)
2.00
1.60
1.20
0.80
0.40
0.00
0
0.064
0.32
1.6
8
40
200
DRUG CONCENTRATION (UM)

Fig.8

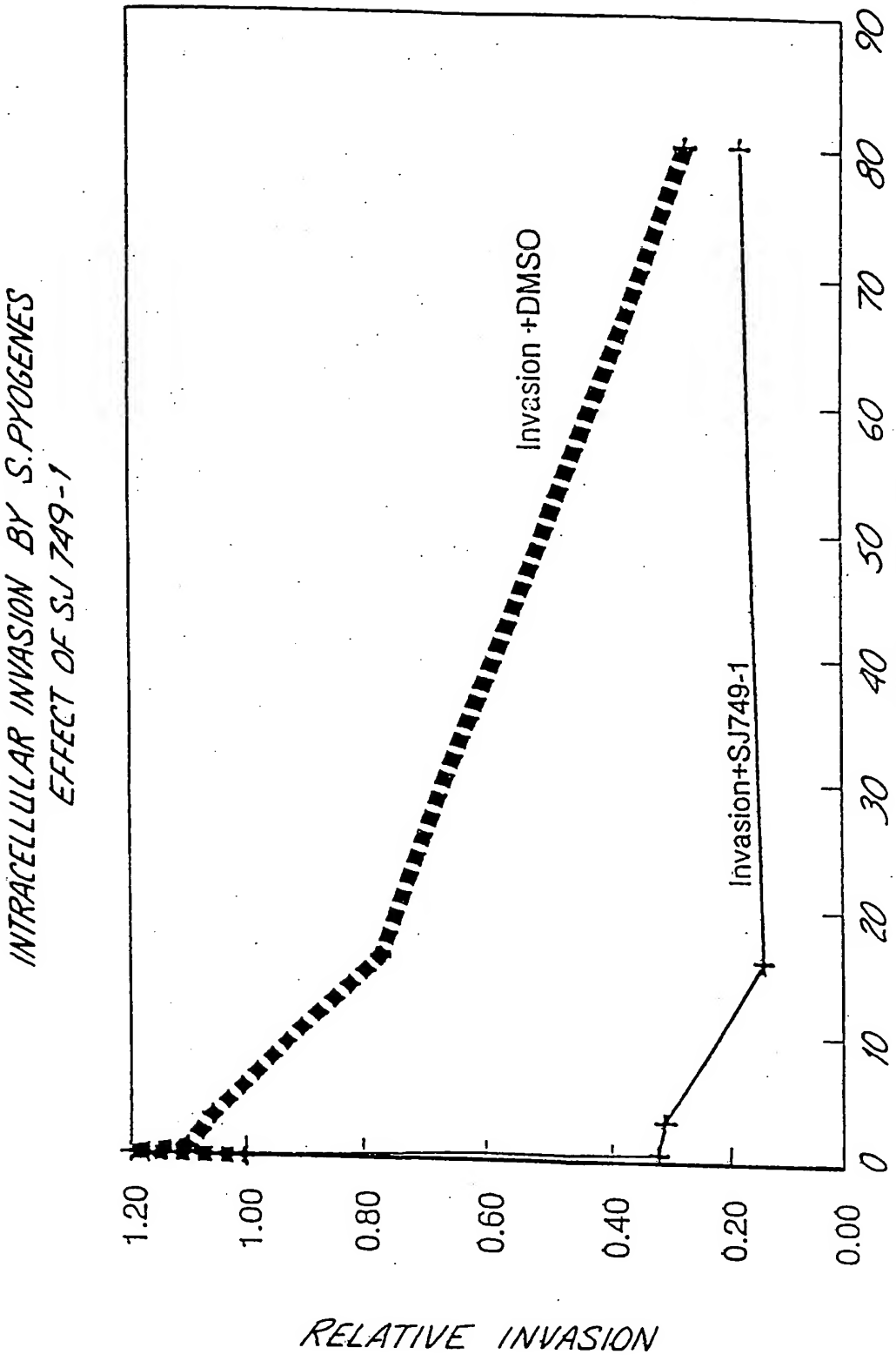

INTRACELLULAR INVASION BY S.PYOGENES
EFFECT OF SJ 749-1
Invasion +DMSO
Invasion+SJ749-1
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
10
20
30
40
50
60
70
80
90
RELATIVE INVASION
CONCENTRATION
SJ749-1 (uM)

Fig. 9

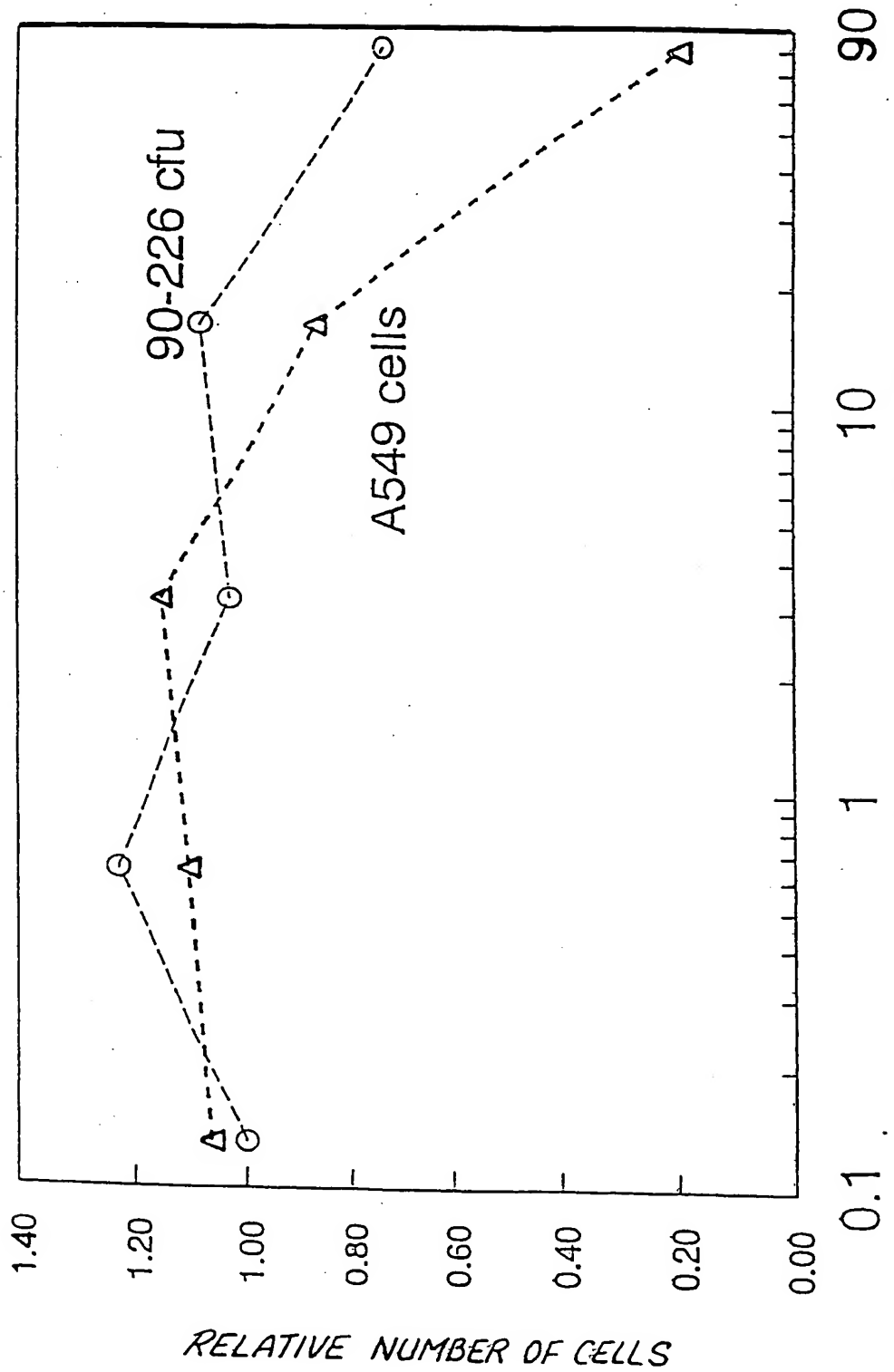

90-226 cfu
A549 cells
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
RELATIVE NUMBER OF CELLS
0.1
1
10
90
CONCENTRATION SJ749-1(uM)

Fig. 10

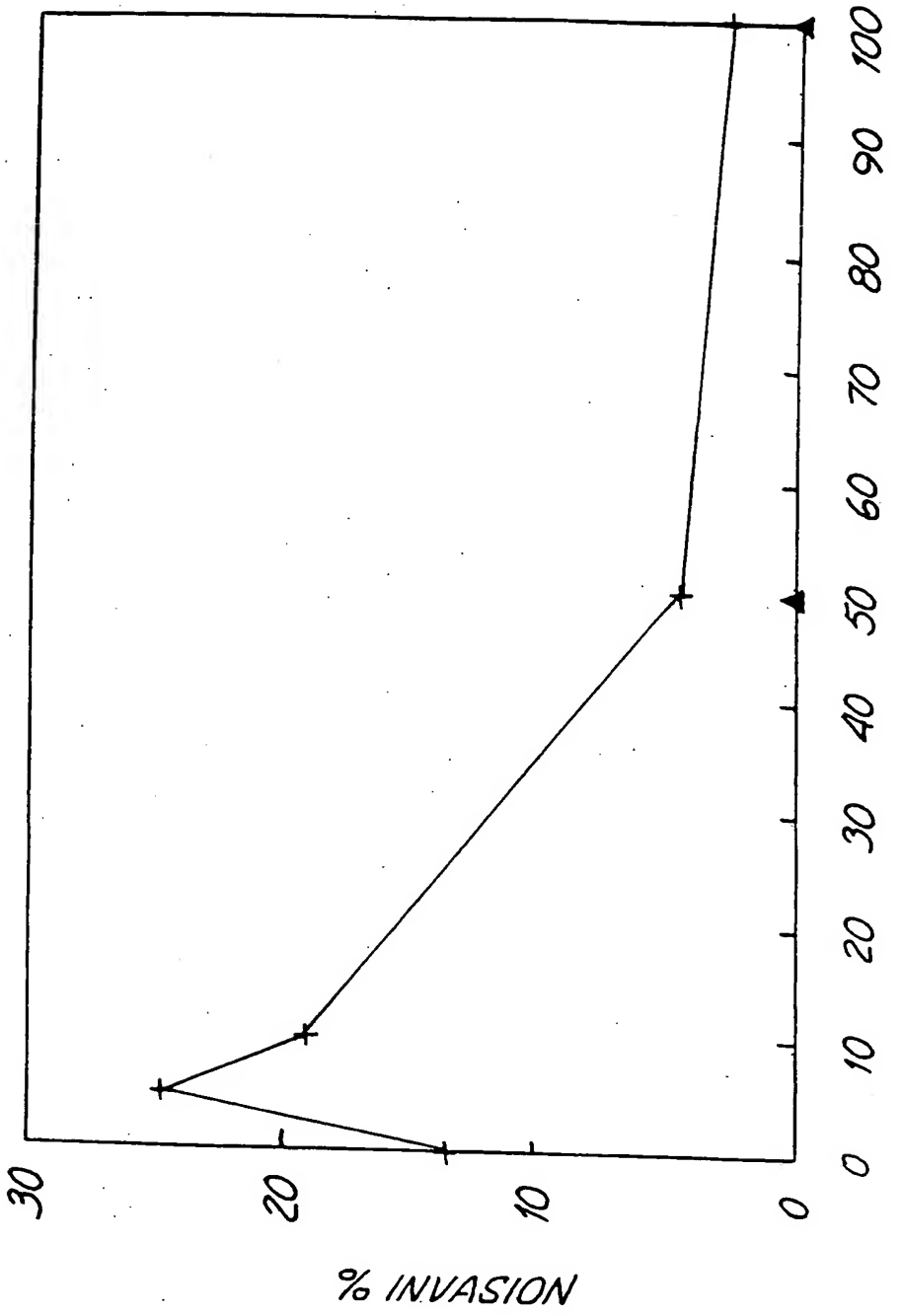

30
20
10
0
% INVASION
0
10
20
30
40
50
60
70
80
90
100
DRUG CONCENTRATION (UM)

Fig. 11

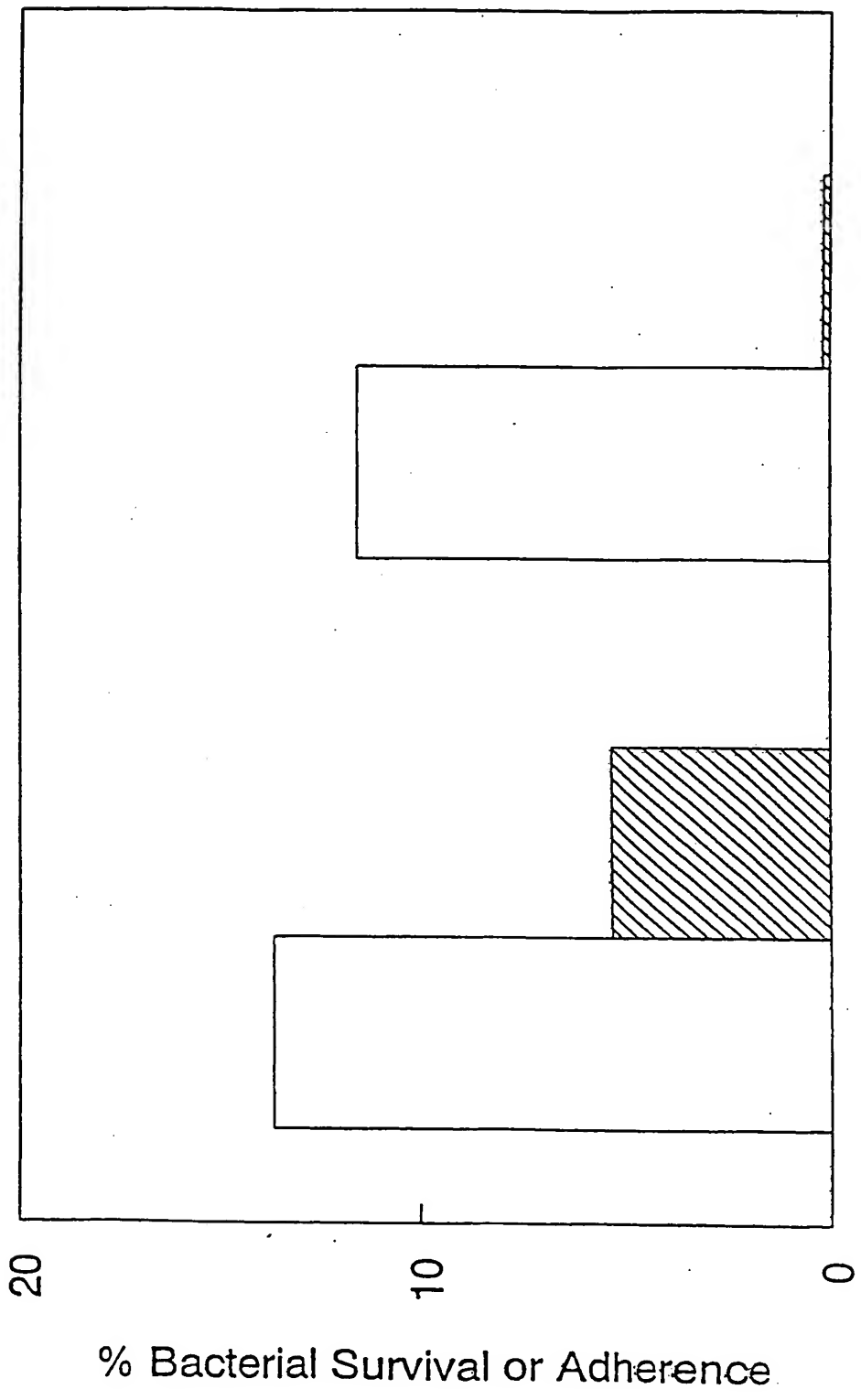

Invasion
Adherence
20
10
0
% Bacterial Survival or Adherence

Fig. 12

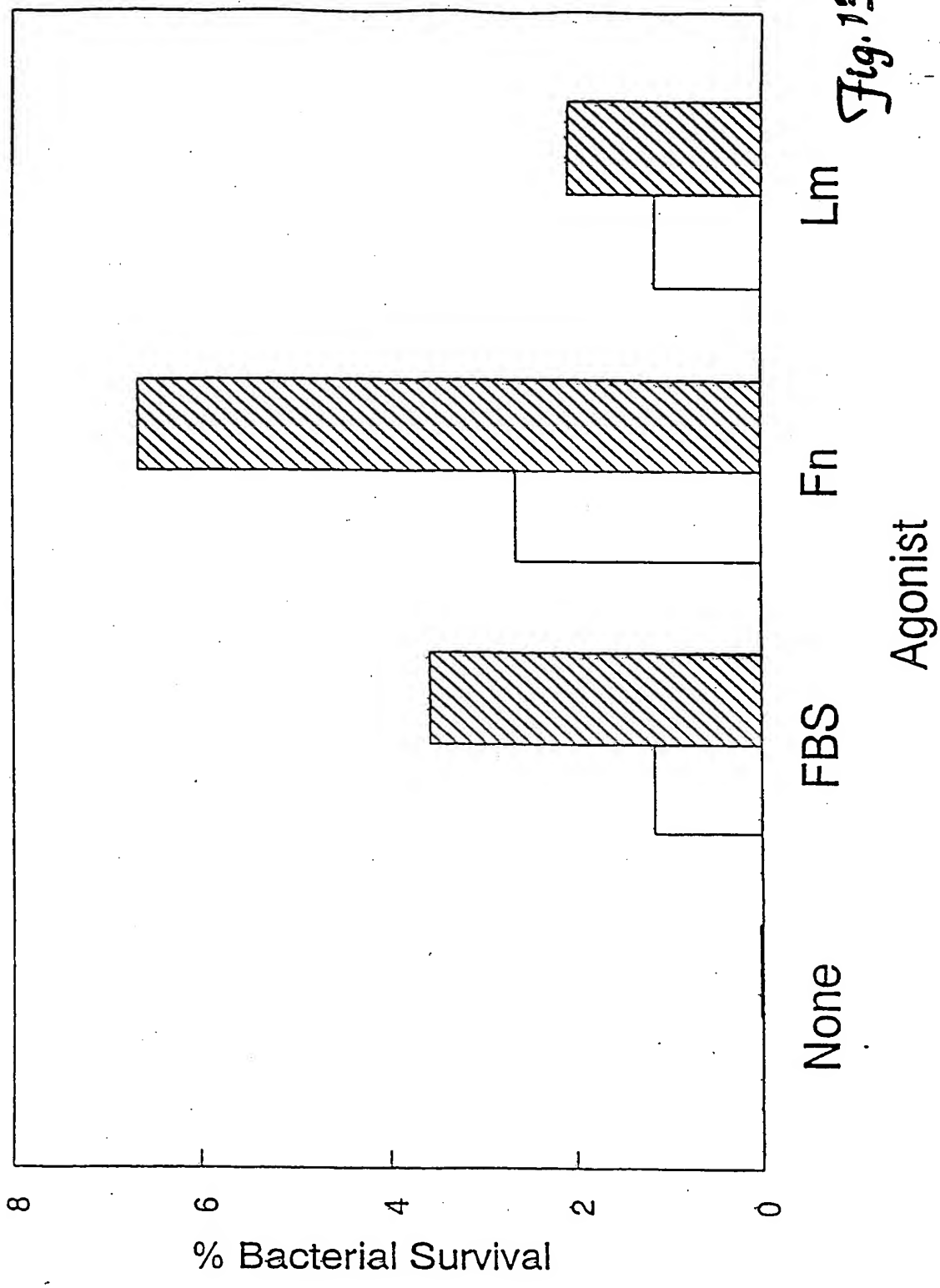


Fig. 13

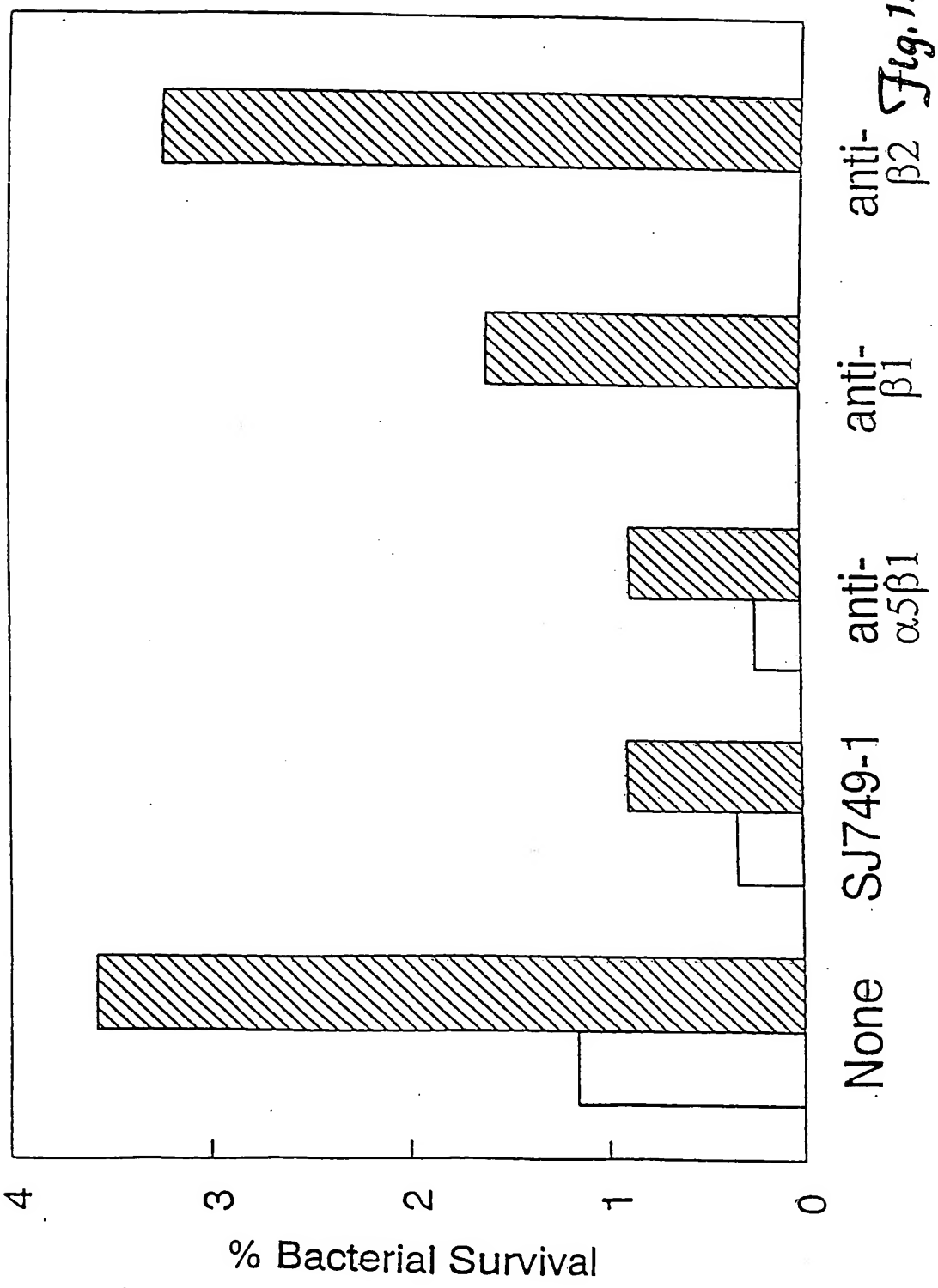

4
3
2
1
0
% Bacterial Survival
None
SJ749-1
anti-α5β1
anti-β1
anti-β2
ANTAGONIST

Fig. 14

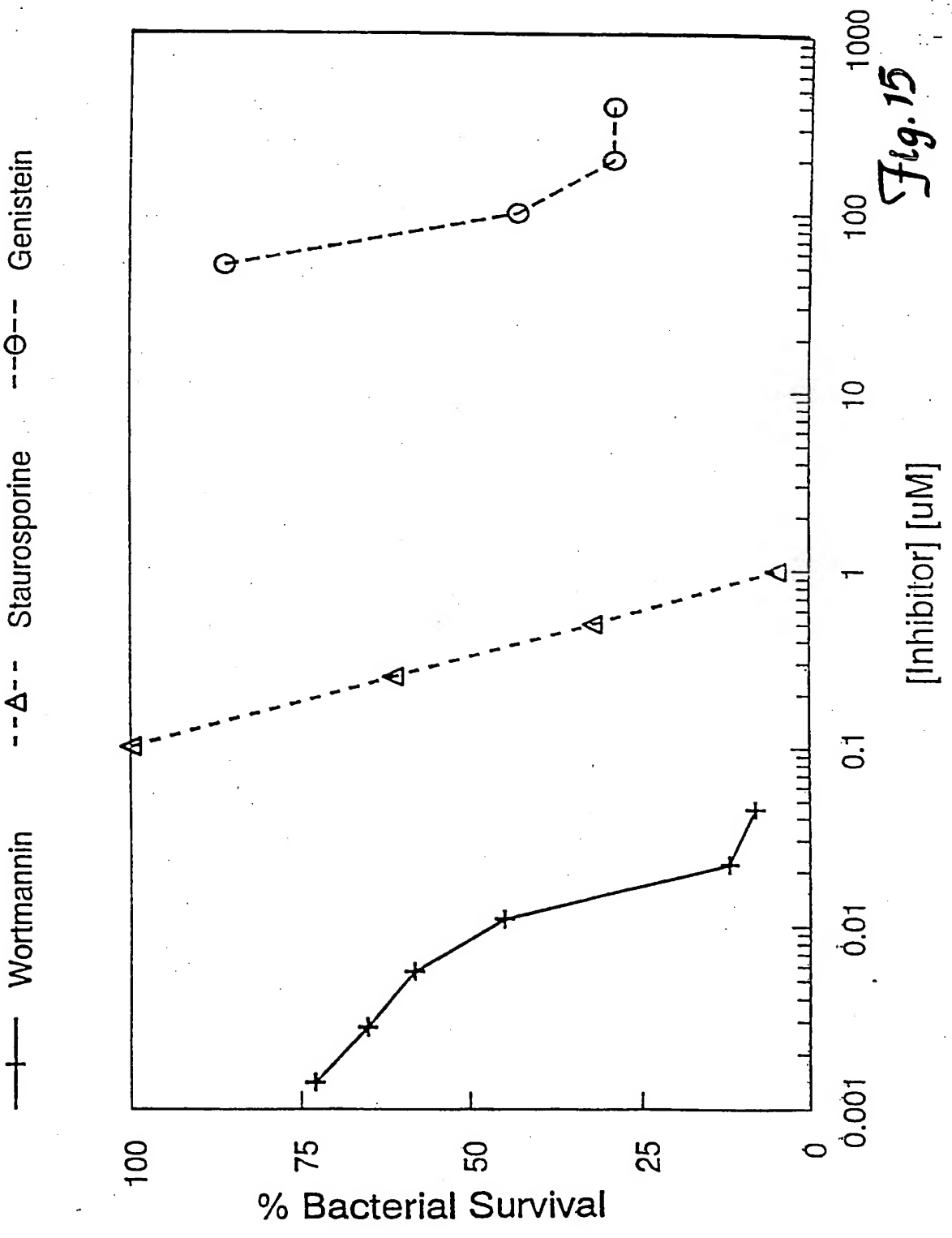

Wortmannin
Staurosporine
Genistein
100
75
50
25
0
% Bacterial Survival
0.001
0.01
0.1
1
10
100
1000
[Inhibitor] [uM]

Fig. 15

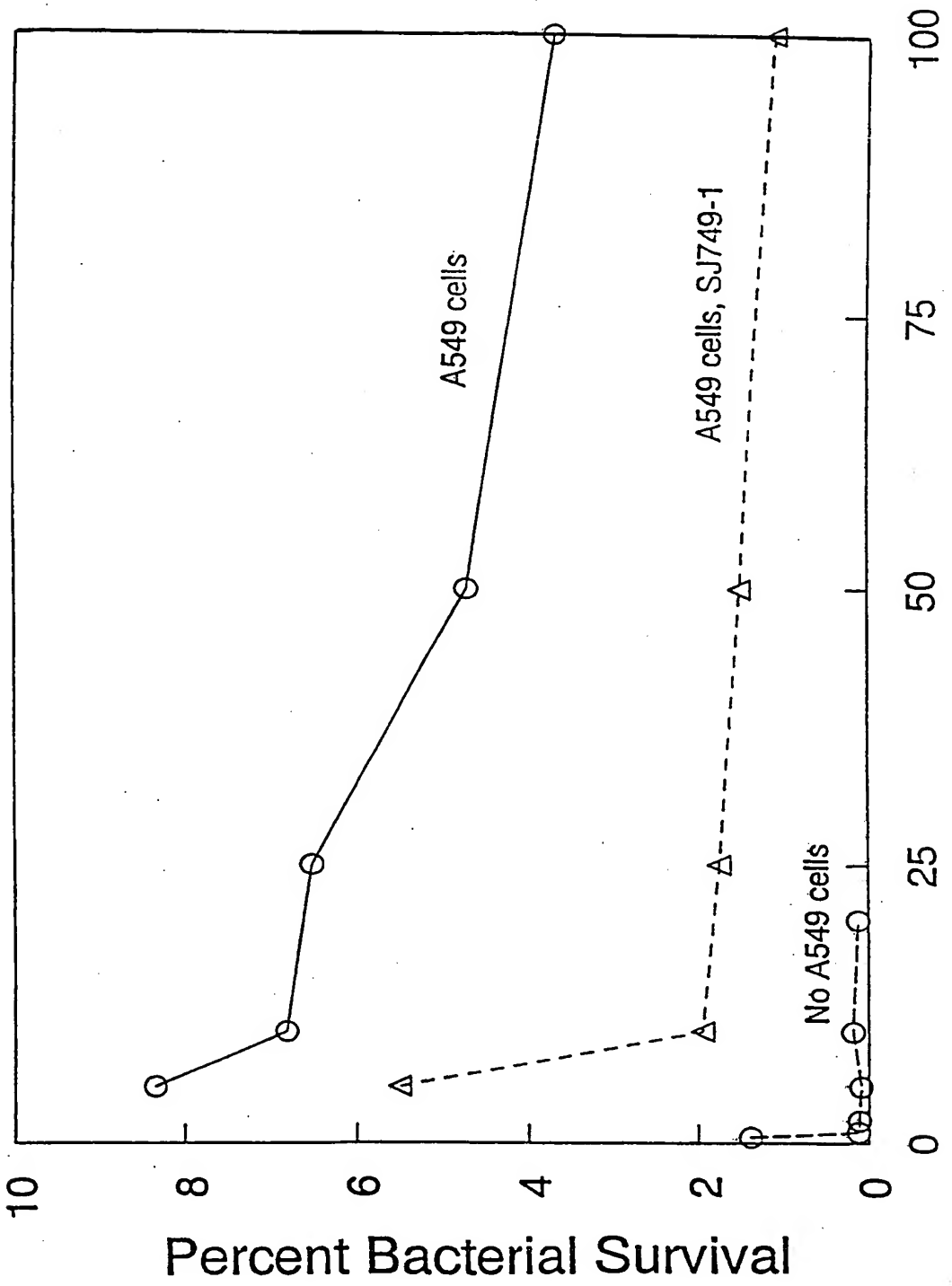

A549 cells
A549 cells, SJ749-1
No A549 cells
0
25
50
75
100
Ug/ml Penicillin
10
8
6
4
2
0
Percent Bacterial Survival

Fig. 16

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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A3

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60/049,124 10 June 1997 (10.06.97) US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GW, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(88) Date of publication of the international search report:
4 March 1999 (04.03.99)

(54) Title: FIBRONECTIN ANTAGONISTS AS THERAPEUTIC AGENTS AND BROAD-SPECTRUM ENHANCERS OF ANTIBIOTIC THERAPY

(57) Abstract

The invention is directed to therapeutic use of fibronectin antagonists to inhibit microbial intracellular invasion of or adherence to host mammalian cells. Co-administration of the inhibitory compound with an antibiotic, such as penicillin, that inefficiently permeates mammalian cell membranes increases the efficacy of the antibiotic therapy.

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INTERNATIONAL SEARCH REPORT

Internati Application No
PCT/US 98/12010

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/39

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 38426 A (THE DU PONT MERCK PHARMACEUTICAL COMPANY) 5 December 1996 cited in the application see the whole document	1-22
Y	US 5 635 477 A (DEGRADO W.F.) 3 June 1997 cited in the application see the whole document	1-22
Y	MOLINARI G ET AL: "The fibronectin -binding protein of Streptococcus pyogenes, SfbI, is involved in the internalization of group A streptococci by epithelial cells." INFECTION AND IMMUNITY, (1997 APR) 65 (4) 1357-63, XP002085309 see the whole document	1-22

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 November 1998	08/12/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter. ..onal application No.
PCT/US 98/ 12010

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-15 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internati Application No
PCT/US 98/12010

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9638426 A	05-12-1996	AU 6024396 A	18-12-1996
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